β-catenin: A friend or foe in liver pathobiology?

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Hepatocellular carcinoma (HCC) is a leading cause of cancer mortality worldwide due to poor prognosis and limited therapeutic options. Point mutations affecting phosphorylation sites such as serine-45 in β-catenin gene are evident in around 30% of HCC. We developed a transgenic mouse that expresses Ser45 mutated β-catenin (TG) in hepatocytes. While this mutation did not induce spontaneous tumorigenesis, it promoted diethylnitrosamine (DEN)-induced HCC through cyclin-D1 overexpression and other factors.

The Wnt/β-catenin signaling is important in stem cell self-renewal. The adult progenitor cell of the liver, or oval cells that emanate from atypical ductular proliferation (ADP), maybe involved in liver regeneration and/or hepatocarcinogenesis and can be observed after exposure to DDC diet, which induces hepatic and biliary injury. When challenged with chronic-DDC diet, β-catenin transgene led to a cellular disparity in the form of increased appearance of atypical hepatocytes (positive for ductular marker A6), which was associated with better resolution of intrahepatic cholestasis. We also utilized DDC diet in conditional β-catenin knockout mice (KO) that lacked β-catenin in hepatocytes and cholangiocytes. ADP was blunted after short-term DDC feeding in KO mice; however, long-term feeding resulted in gradual increase in ADP, hepatic fibrosis and HCC. Interestingly, the KO livers begin to exhibit periportal β-catenin-positive hepatocytes, which eventually populate the entire livers over the course of this process.

Finally, we explored targeting of the Wnt pathway with pegylated interferon-α2A (Peg-IFN). We found that Peg-IFN decreased β-catenin activity in mouse liver and several human hepatoma cell lines. The mechanism seemed to be at least partly due to upregulation of a nuclear export factor, RanBP3.

Thus, this study characterizes an animal model utilizing β-catenin mutation, which is evident in HCC patients. DEN-exposure in these animals led to HCC development, thus

providing a valuable tool to study mechanisms of hepatocarcinogenesis and providing a model to test therapeutic inhibition of β-catenin by agents such as peg-IFN and others. Our studies also provide evidence that Wnt activation may resolve intrahepatic cholestasis. Finally, we show that chronic damage to the liver in KO led to appearance of β-catenin-positive hepatocytes, which continued to proliferate and in the face of continued injury and fibrosis, led to development of HCC, which is also relevant clinically.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

PREFACE

I would like to take the opportunity to thank all of the people who have been helpful and supportive during my time conducting the research presented here. First, I want to thank all of the members of the Monga Lab both past and present who were around during my time in the lab. They have always been a great source for answering questions and giving me technical input while troubleshooting experiments. They have also made the lab a fun and happy place to spend much of my time. I also want to thank my thesis committee for all of their help and insight over the last four years providing different perspectives on how to interpret my work as well as new ideas for moving forward with my projects.

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Lastly, I want to thank my amazing mentor Dr. Satdarshan Monga. Nobody has had a greater hand in developing me as a scientist guiding me from a fairly inexperienced student in the lab to someone capable of developing my own experiments and taking my work and converting it to a form suitable for publication or grant application. He has also put in significant

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1.0 INTRODUCTION

1.1 WNT SIGNALING PATHWAY

1.1.1 Introduction to the Wnt Pathway

Wnts were first described more than 30 years ago when a recessive mutation in the gene *wingless(wg)* in *Drosophila melanogaster* resulted in abnormal wing development (1). It was further identified that mutations in *wg* affected proper segmentation and polarity in Drosophila (2). Int-1, a gene with significant homology and evolutionary origins to *wg*, was found near integration sites of mouse mammary tumor virus (MMTV) linking the protein to cancer (3, 4). A combination of the two gene names led to the commonly used name "Wnt". Further studies described Wnt as a family of secreted glycoprotein ligands which bind to a family of 7-transmembrane receptors called Frizzled initiating a multitude of downstream effects important in development, growth, and disease. The primary downstream effector of this pathway is β-catenin, although other non-canonical pathways have been described (reviewed in (5)).

1.1.2 Canonical Wnt Pathway

At steady state, free cytoplasmic β-catenin is targeted for ubiquitin-mediated degradation by a well-described destruction complex that consists of Axin, adenomatous polyposis coli (APC),

casein kinase 1 (CK1), and glycogen synthase kinase 3β (GSK3β). A series of serine (Ser) and threonine (Thr) phosphorylation events are involved in this process in association with the destruction complex (Fig. 1). First, CK1 phosphorylates Ser45 of β-catenin acting as the rate limiting step in this process (6). Following Ser45 phosphorylation, GSK3β phosphorylates β-catenin at Ser33, Ser37, and Thr41 (7). Phosphorylated β-catenin then undergoes ubiquitination mediated by βTrCP and is degraded by the proteasome (8).

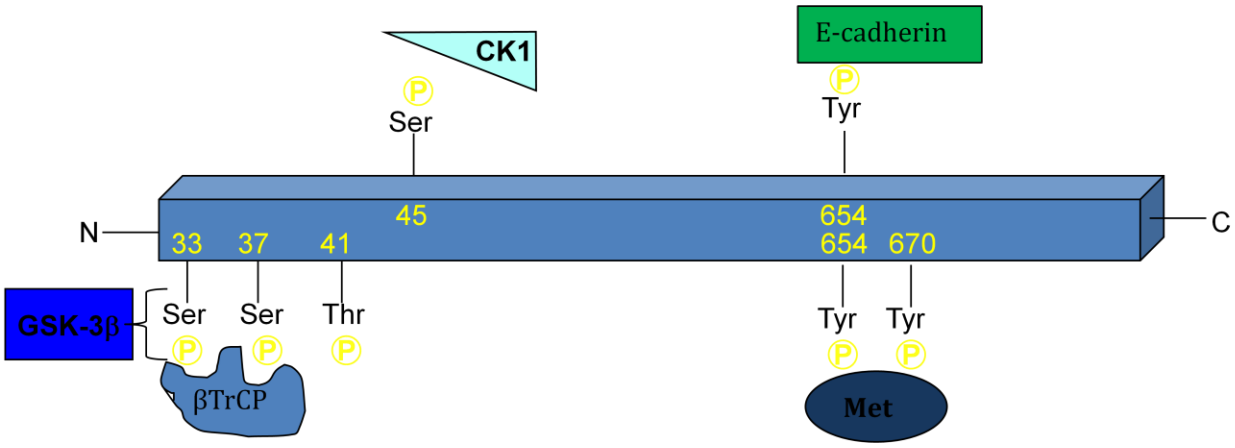


Figure 1: Phosphorylation of β-catenin

CK1 phosphorylates β-catenin at Ser45 followed by phosphorylation of Ser33, Ser37, and Thr41 by GSK-3β. Phospho-serine 33 & 37 are recognized by βTrCP inducing ubiquitin-tagging of the protein. Other important phosphorylation sites include Tyrosine (Tyr)654 and Tyr670 which are important for the interaction between β-catenin and E-cadherin or Met.

Canonical activation of the pathway is defined by cytoplasmic accumulation of free β-catenin and subsequent nuclear translocation leading to expression or repression of downstream targets (Fig. 2). Binding of Wnt ligand to the receptor frizzled induces the formation of a ternary complex with low density lipoprotein receptor-related protein 5 (LRP5) or LRP6 (9, 10). Frizzled then recruits dishevelled to the plasma membrane via its PDZ domain and binds via its

DIX domain (11, 12). Dishevelled is involved in relocating the destruction complex to the membrane where GSK3β phosphorylates LRP6 in an Axin-dependent manner (13-15). Although the molecular mechanism is yet known, phosphorylation of LRP6 leads to an inhibition of β-catenin phosphorylation by GSK3β and subsequent release from the degradation complex.

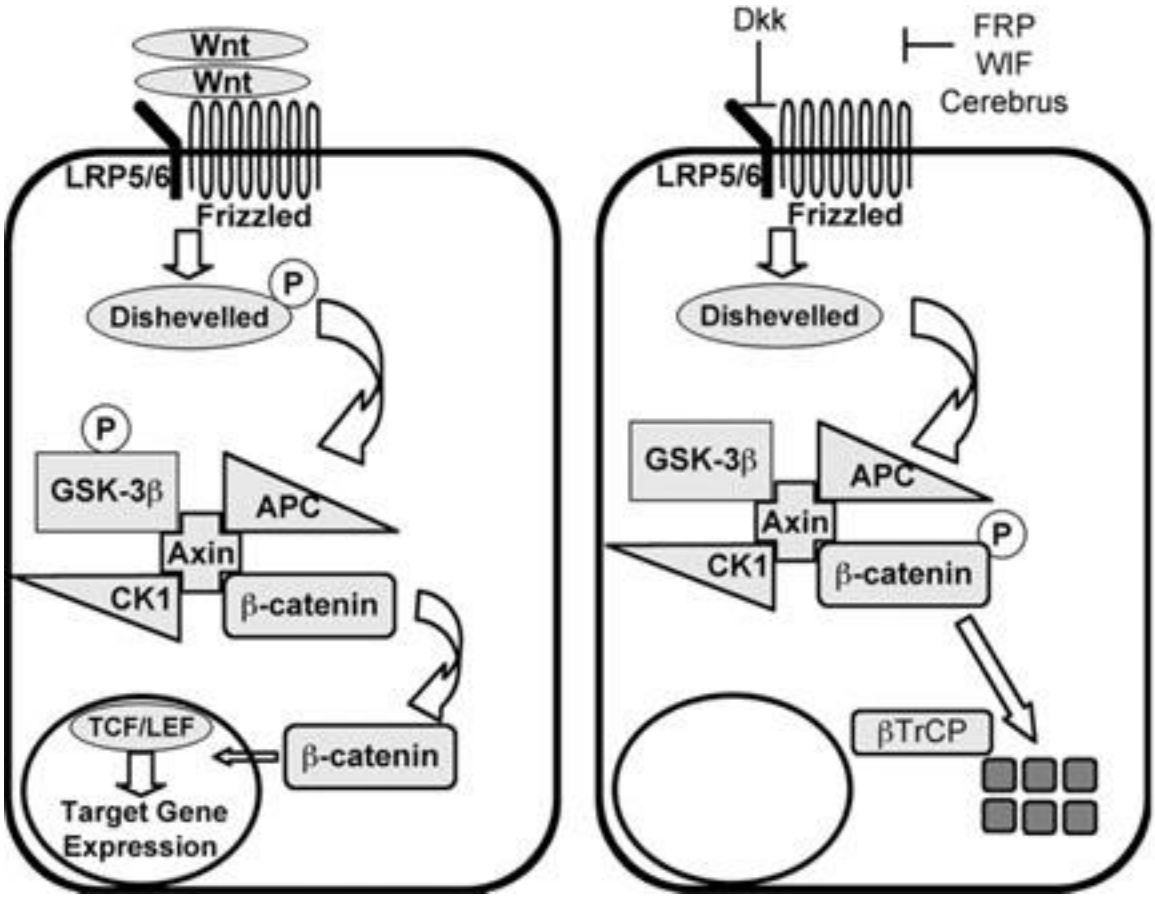


Figure 2: Canonical Wnt Signaling Pathway.

Left: Presence of Wnt induces hypophosphorylation of β-catenin at specific serine/threonine residues to induce its nuclear translocation and activate target gene expression. Right: Absence of Wnt or presence of inhibitors that prevent Wnt binding to its receptor or dimerization of co-receptors enable activated kinases such as GSK3β to phosphorylate β-catenin, which, with the help of Axin and APC, undergoes proteasomal degradation. Abbreviations: APC, adenomas polyposis coli; CK, casein kinase; FRP, frizzled-related protein; GSK, glycogen synthase kinase 3 B; LRP, low-density lipoprotein receptor related protein; TCF, T-cell factor; TrCP-transducin repeat-containing protein.

How β-catenin gets into the nucleus is yet unclear as it lacks any recognizable nuclear localization sequence (NLS). One possibility is that LEF/TCF, a primary binding partner for β-catenin in the nucleus, is involved in this process. Indeed, over expression of LEF/TCF promotes nuclear localization of β-catenin (16, 17). However, nuclear shuttling of β-catenin by LEF/TCF has yet to be definitively shown and other studies have reported nuclear translocation of β-catenin independent of LEF1 (18, 19). Another possibility is that β-catenin may be able to directly interact with components of the nuclear pore complex. Interestingly, β-catenin competes with importin-β for binding to components of the nuclear pore complex (20, 21). Future studies will likely elucidate the true nature of β-catenin's nuclear shuttling.

While it is unclear how free β-catenin translocates into the nucleus, once it is there it primarily partners with members of the lymphoid enhancing factor/t-cell factor (LEF/TCF) family initiating transcription of target genes (22, 23). β-catenin is not capable of binding directly to the DNA so it is dependent on transcription factors such as TCF to mediate its transcriptional control. In the absence of β-catenin, TCF partners with Groucho and histone deacetylases to act as a transcriptional repressor of downstream genes (24, 25). Once β-catenin is in the nucleus, it can directly replace Groucho in binding to TCF (26).

To shut off β-catenin transcriptional activity, several factors act to export β-catenin from the nucleus. One well-described interaction is with APC which binds to β-catenin in the nucleus and provides a nuclear export sequence to promote removal from the nucleus (27-29). This event happens in a CRM-1 dependent manner. Another more recently described path for nuclear export of β-catenin involves Ran binding protein 3 (RanBP3). RanBP3 can bind to both full-length and N-terminally deleted β-catenin and induce its nuclear export in a CRM-1 independent manner

(30). The fact that RanBP3 can export mutated β-catenin has clear implications for cancers that harbor similar mutations.

1.1.3 Target Genes of Canonical Wnt Signaling

The Wnt pathway is capable of regulating transcription of a wide array of genes and does so in a cell and context specific manner. The genes regulated by β-catenin are involved in diverse cellular functions including cell cycle, proliferation, apoptosis, differentiation, cell adhesion, metabolism, and the immune response. Some of the primary targets are described here, but a regularly updated full listing of target genes is provided on the Wnt homepage: http://www.stanford.edu/~rnusse/wntwindow.html.

First of all, several signaling components of the Wnt pathway itself are regulated by β-catenin. These pathway members include LEF1, Axin-2, Dkk-1, Frizzled-7, and β-Trcp (31-35). Several of these targets are negative regulators of the Wnt pathway indicating a role for negative feedback in the control of activity of this pathway. Several cell cycle and proliferation related factors are also controlled by β-catenin. The most prominent of this group is cyclin D1, which is upregulated by Wnt/β-catenin activity (36, 37). However, one report has disputed this finding suggesting that cyclin D1 is not an immediate target of β-catenin (38). Oncogene expression may also be controlled by β-catenin. One well-known oncogene target is c-myc which was identified in human colon cancer cell lines (39). Other common targets include survivin, Met, MMP7, and Jagged, many of which were also identified from human colon cancer cell lines (40-43). There are also a group of targets which are specific to the liver. These targets include glutamine synthetase, various cytochrome P450s, glutathione-S-transferases (GSTs), leukocyte cell-derived chemotaxin 2 (LECT2), and regucalcin (44-48).

1.1.4 β-catenin and the Adherens Junction

In addition to its role as the downstream effector of the Wnt pathway, β-catenin has a well-described and essential role in the formation of the adherens junction (AJ) at the cellular membrane (Fig. 3). E-cadherin, the primary adhesive factor in the AJ, binds to β-catenin during its transport to the membrane through the secretory pathway (49). β-catenin binds to the intracellular C-terminal region of E-cadherin and once at the plasma membrane forms a link to α-catenin and the actin cytoskeleton (50-52). This interaction is essential for maintenance of the AJ and is regulated by phosphorylation events. Indeed, Ser/Thr phosphorylation of β-catenin and E-cadherin by casein kinase II (CKII) promotes interactions between the two along with α-catenin at the membrane (53, 54). On the other hand, tyrosine phosphorylation disrupts these interactions leading to an increase in free cytoplasmic β-catenin (55, 56). More specifically, it was found that phosphorylation of β-catenin at Tyr-654 is the key event in disrupting the connection with E-cadherin (55).

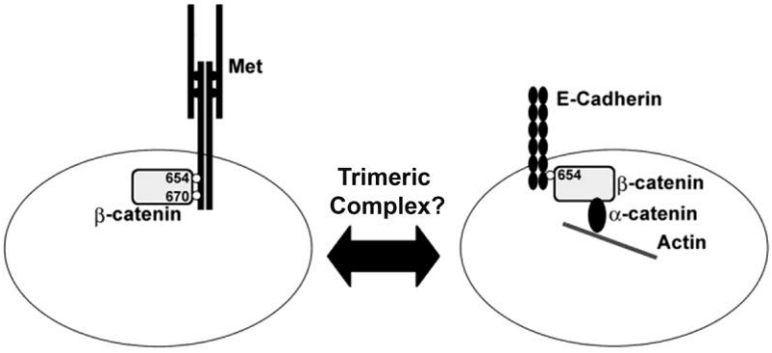


Figure 3 Membranous associations for β-catenin.

Left: β-catenin binds to intra-cytoplasmic tail of β-subunit of c-Met. HGF stimulation incites β-catenin tyrosine-phosphorylation at residues 654 and 670, which induces nuclear translocation and activation of β-catenin. Right: β-Catenin acts as a connector between intra-cytoplasmic tail of E-cadherin and α-catenin and on tyrosine-phosphorylation at 654, negatively regulates this adhesive function, making cells more motile.

While the interaction between β-catenin and E-cadherin is important for cell-cell adhesion, it may play a key tumor suppressive role. The observation that an overall loss of E-cadherin or a decrease in its membranous localization is associated with tumor progression and metastasis, as well as both somatic and germline mutations in E-cadherin in human tumors, is reported in multiple studies (57-61). Given that E-cadherin localizes β-catenin at the membrane, it is reasonable to hypothesize that a loss of E-cadherin in these tumors along with some alteration in β-catenin degradation provides an increased pool of free β-catenin to promote cell proliferation and tumor progression. Studies in embryonic development clearly show that over expression of E-cadherin decreases β-catenin transcriptional activity, and a loss of E-cadherin increases β-catenin signaling (62-64). However, loss of E-cadherin does not necessarily always equate to an increase in β-catenin activity and tumor progression (65-67). It is feasible to consider that E-cadherin acts as a "sponge" at the membrane to "soak up" excess β-catenin; and that in the setting of mutation or loss of E-cadherin, an increase in free β-catenin makes the cell more sensitive to stimuli promoting Wnt pathway activity. Future studies will be essential to definitively address this hypothesis.

1.1.5 β-catenin and the HGF receptor Met

Hepatocyte growth factor (HGF) mediated activation of β-catenin has been described. The receptor for HGF is Met which is capable of tyrosine phosphorylation of multiple effectors including GRB2, SHC, SRC, and PI3K ultimately playing a role in cell growth, motility, proliferation, and survival. Another well-defined interaction for Met is that it directly binds to β-catenin (Fig. 3). Following binding of HGF, Met is involved in phosphorylation of β-catenin at tyrosine residues 654 and 670 with subsequent nuclear translocation of β-catenin (68-70). Prior

to HGF stimulation, β-catenin exists in a complex with Met at the membrane which becomes dissociated upon binding of HGF. It is likely that such growth factor stimulation of β-catenin plays a role in various cancers. Indeed, Met and β-catenin cooperate to promote proliferation and invasiveness of colorectal cancer cells via a positive feedback loop (71). Likewise, expression of the Met gene is increased by Wnt stimulation in dysplastic aberrant crypt foci (41). Thus, a positive feedback loop can occur where HGF stimulates nuclear translocation of β-catenin which can promote increased transcription of the receptor Met. Increased levels of Met then further potentiates this circuit providing more receptor for HGF to induce its own downstream targets, as well as further promote the pro-proliferative effects of activated β-catenin. Given that tyrosine phosphorylation of residue 654 is a common feature shared between dissociation of β-catenin from Met and dissociation of β-catenin from E-cadherin, it is believed that the two may not be mutually exclusive. It is possible that a trimeric complex forms between β-catenin, E-cadherin, and Met bringing together the two cellular functions of adhesion and growth factor signaling with β-catenin as the central player. Future work will need to address whether or not such an interaction exists and what, if any, implications it may have in the setting of cancer.

Colorectal cancer is not the only tumor for which an association between HGF signaling and β-catenin has been observed. In invasive breast carcinoma, a significant correlation exists between expression of c-met and abnormal β-catenin expression implicating crosstalk between the two in breast cancer (72). The presence of abnormal β-catenin suggests poor prognosis in many tumors; however, abnormal expression of β-catenin and c-met in breast cancer tumors from this study was a predictor of a favorable outcome. A connection has also been made in liver cancer where cooperation between activated Met and constitutively active forms of β-

catenin induced development of HCC in mice (73). While many of the pro-proliferative effects of β-catenin are mediated by expression of cyclin D1, Met and β-catenin cooperation in liver tumor development seems to be independent of cyclin D1 expression (74). Such cooperation between signaling pathways may also be important in human tumors as an association between phosphorylated Met and mutated β-catenin was observed in a subset of human HCC (73). Currently, the best available chemotherapy for liver cancer is sorafenib, a multiple tyrosine kinase inhibitor (75). Given the capacity for both EGF and HGF tyrosine kinase pathways to transactivate β-catenin, it is plausible that sorafenib may have an indirect effect on β-catenin by downregulating the pathways that transactivate it.

1.2 BETA-CATENIN IN LIVER DEVELOMPENT, GROWTH, AND REGENERATION

1.2.1 Liver Development

Liver derivation from the foregut endoderm occurs around somite stage 5-6 as a result of signaling from mesoderm in the form of fibroblast growth factors (FGFs) and bone morphogenetic protein-4 (BMP4), both of which are incidentally downstream targets of the Wnt pathway (76, 77). Based on the role of β-catenin in HCC, our group decided to apply the oncofetal paradigm to investigate the role of Wnt/β-catenin pathway in hepatic development. We were the first to discover the role of β-catenin during hepatic development. When whole livers from E10 were cultured with antisense oligonucleotides against the β-catenin gene (Ctnnb1), there was a noteworthy decrease in β-catenin protein, cell proliferation, and survival (78).

Conversely, over expression of constitutively active β-catenin in the developing liver was shown to lead to a three-fold increase in liver size and an expansion of the hepatocyte precursor cell population (79). In another study, we reported temporal expression of β-catenin during mouse liver development with high levels at embryonic day 10 (E10)-E14 (80). During this period in liver development, nuclear/cytoplasmic localization in hepatoblasts correlated with increased cell proliferation. This effect on cell proliferation might be mediated by downstream targets that are playing a role in cell cycle such as cyclin D1. Thus, β-catenin is likely playing an important role during early liver development in hepatocyte expansion via its transcriptional co-activator role. Interestingly, after E15 stage, an overall decrease in total β-catenin levels was observed when it began localizing chiefly to the hepatocyte membrane (80). This membranous localization and functional complex with its partners, especially E-cadherin, might be a hallmark of acquisition of hepatocytic maturation and polarity. Indeed, antisense ablation of β-catenin promoted a more immature cell type that continued to coexpress stem cell and mature hepatocyte markers (78). These observation were further substantiated by a pleiotropic phenotype associated with Foxa3-Cre-mediated β-catenin conditional deletion (81). Additionally, studies utilizing matrigel in primary hepatocyte cultures, showed an increase in membranous complexes as a part of the differentiation process (82). This indicates that hepatocyte maturation might be a function of cell-cell adhesion properties of β-catenin with some contribution from its transcriptional coactivator function. The latter function might be associated with the function of β-catenin in regulating expression of genes involved in hepatocyte maturation, such as the cytochrome P450s, shown recently to be tentative targets of the Wnt/β-catenin pathway in liver (83, 84).

What regulates β-catenin activity during liver development at different stages is only beginning to be resolved. A recent study identified active mesodermal Wnt2b signaling at these

stages, which for the first time documents an important role of Wnt signaling as a positive regulator of liver specification and induction in zebrafish (85). Additional studies would be of essence to examine if Wnt2b or additional Wnts might be upstream of the factors known to be critical for hepatic induction. Similarly, other ligands for β-catenin activation such as Wnt9a and FGF have been reported. While no direct evidence is available, Met knockouts show embryonic lethality due to defective liver development. Based on the known β-catenin-Met interaction and the resemblance of the phenotype between these embryos and β-catenin conditional nulls, it is conceivable that HGF/Met may be an important regulator of β-catenin during hepatic development as well.

β-catenin also has a role in biliary specification during liver development. β-catenin antisense ablation in E10 liver cultures led to an absence of CK19-positive biliary cells (78). Conversely, growth in Wnt3a-conditioned media showed survival and proliferation of predominantly CK-19 positive cells as compared to the control media or Wnt3a-conditioned media containing soluble Frizzled-related protein-1 (sFRP1), a Wnt inhibitor (86). Similarly, deletion of β-catenin by FoxA3-driven cre recombinase led to paucity or complete absence of CK-19 positive primitive bile ducts (81). Moreover, activation of β-catenin in the embryonic liver via deletion of Apc led to a partial commitment toward the biliary lineage in hepatoblasts prior to embryonic lethality due to failure of hepatocytes differentiation (87). Interestingly, transplantation of E14.5 livers from these mice into adult recipients allowed for completion of biliary differentiation and the formation of mature bile ducts. Thus, the evidence indicates that β-catenin plays an important role in biliary development.

1.2.2 Normal Liver Growth

The first month after birth in mouse is characterized by a spurt in hepatic growth. Increased β-catenin activity is observed during this stage and correlates well with levels of hepatocyte proliferation (88). Mice over expressing a stable-mutant or full-length-β-catenin displayed a 3-4-fold or 15% increase in liver size due to increased hepatocyte proliferation, respectively (89, 90). Interestingly, none of the conventional targets appear to be upregulated in any of these transgenic mice. Conversely, the β-catenin conditional null mice show decrease in their liver size within the first month after birth, where decreased basal hepatocyte proliferation is evident. There is also decreased cyclin D1 in the β-catenin-deficient livers and this deficit became more pronounced during states requiring de novo cyclin D1 synthesis such as during liver regeneration following partial hepatectomy (84). These observations suggest a clear role of β-catenin in normal liver growth.

1.2.3 Liver Regeneration

In light of β-catenin's role in liver growth, multiple studies have shown its role in optimal liver regeneration. Much of this endeavor has utilized the two-thirds partial hepatectomy model, which is an in vivo system that has been widely used in the study of physiologic and regulated liver growth. In rats, following partial hepatectomy, β-catenin protein is increased within minutes of hepatectomy as mediated by an epigenetic or post-translational mechanism that is at least initially transcription independent (91). While this increase is transient lasting less than 15 minutes, the accompanying β-catenin nuclear translocation is sustained and can still be found in the nucleus for up to 48 hours after hepatectomy. β-catenin knockdown through the use of

antisense in this model led to a significant decrease in liver weight to body weight ratio due to decreased cellular proliferation (92). Additionally, it is evident that HGF signaling is an important player in early liver regeneration and an increase in levels of HGF and activation occurs early following partial hepatectomy, which may have consequences on the Met-β-catenin complex as well (93). These studies suggest that β-catenin might be an early player in regeneration potentially initiating a cascade of events that are important for successful liver regeneration. Indeed, some downstream targets of this pathway such as cyclin D1, c-myc, uPAR, MMPs, EGFR, and others are known to be upregulated concurrently during nuclear localization of β-catenin (94). Similar findings were recently reported in mice, where conditional β-catenin knockout mice exhibit a 24 hour delay in peak regeneration following hepatectomy, at which time, the hepatocyte proliferation came back with a 'vengeance' (84, 95). This study identifies factors such as IL-6, PDGFRα, decorin and others that might be compensating for β-catenin loss and would need to be confirmed by additional studies.

1.3 HEPATIC TUMORIGENESIS

1.3.1 Hepatoblastoma (HB)

Hepatoblastoma is the most common malignant liver tumor found in pediatric populations, with the incidence being highest in populations suffering from familial adenomatous polyposis coli (APC) (96). Nuclear and cytoplasmic localization of β-catenin were reported in 90-100% of all hepatoblastomas, familial and sporadic, due to mutations in APC, Ctnnb1, Axin1, and Axin2, clearly demonstrating its role in both types of hepatoblastoma (97-99). In a recent study,

microarray analysis was used to examine and identify two different subclasses of hepatoblastoma (100). Aberrant activation of β-catenin is a major component in both subtypes with the stratification between the two groups involving differentiation between tumors in early and late phases of liver development. While both subclasses exhibit over expression of Axin2 and Dkk-1, two downstream targets of β-catenin, they show clear differences in immunolocalization of β-catenin. The more immature subclass (C2) shows predominantly nuclear and cytoplasmic β-catenin, while C1 shows predominantly membranous localization. Additionally, C1 tumors over express downstream targets involved in liver zonation and metabolism, such as glutamine synthetase. These targets were decreased in C2 tumors, whereas myc, survivin, and EpCam were highly expressed. Of further clinical relevance, this study provided a 16 gene signature which could be used to accurately differentiate between the two subclasses and served as a strong predictor of tumor aggressiveness and survival. This study not only has provided insight into the molecular pathogenesis of different subclasses of hepatoblastoma but also has implications in identifying patients who might benefit the most from therapies directed at the Wnt pathway.

1.3.2 Focal Nodular Hyperplasia (FNH)

Focal nodular hyperplasia is a benign tumor of the liver which occurs most commonly in women and is thought to be a hyperplastic response to increased blood flow. While this tumor is the second most common in liver, the molecular basis for its development is currently unknown. A transcriptome analysis revealed activation of the Wnt pathway, albeit in the absence of any mutations in *Ctnnb1* (101). In accordance with this finding, over expression of a primary target of β-catenin, glutamine synthetase (GS), is observed in FNH (102). How β-catenin activation occurs in these tumors is currently unclear as other studies corroborate the observation that

mutations in Ctnnb1 do not occur in FNH (103). Given the absence of mutations in Ctnnb1 in some tumors, it is interesting to note that in a study on the fribolamellar variant of HCC our group found an increase in Tyr654 phosphorylation of β-catenin (104). This suggests an alternate mechanism for tumors to utilize the Wnt pathway in the absence of stabilizing mutations in the gene itself.

1.3.3 Hepatic Adenoma

Hepatic adenomas (HA) are another set of benign liver tumors which occur more frequently in women and these tumors result from proliferation of hepatocytes in an otherwise normal liver (reviewed in (105)). The occurrence of HA has been associated with several factors including the use of estrogen-containing oral contraceptives or steroid anabolic drugs and glycogen storage diseases. Some of the microscopic features of this tumor can include cord of plate-like arrangements of large hepatocytes containing excess fat or glycogen, pseudo-encapsulation due to compression of adjacent hepatocytes, paucity of bile ductules, a decrease in Kuppfer cells, and sinusoidal dilatation. While the tumors are benign, HAs are more prone to hemorrhage. Surgical resection is often indicated to prevent this risk.

The molecular basis for development of HA is the subject of many studies to date. Two genes are commonly mutated in these tumors. First, a significant percentage of HA harbor inactivating mutations in the HNF1α gene (106). Additionally, biallelic inactivating mutations in the HNF1α or Tcf1 gene were identified in 35–50% of HA (107). In the setting of HNF1α inactivation, marked steatosis and excess glycogen accumulation are observed (107-109). HA with HNF1α inactivation display an extremely low risk of malignant transformation. A second subset of HA's show Wnt/β-catenin activation often as a result of mutations in *Ctnnb1* that affect

the degradation of β-catenin protein. Nuclear accumulation of β-catenin in HA ranges from 30% to 46% of tumors (103, 110). Interestingly, these HAs have shown a high propensity for malignant transformation as 46% of tumors harboring mutation of *Ctnnb1* progress to HCC (106). A third subset of HA has been described which is associated with a significant inflammatory infiltrate (111). This subset also exhibits an increased risk for progression to HCC and all such transformation occurred in the setting of CTNNB1 mutation.

1.3.4 Hepatocellular Carcinoma (HCC)

HCC is the most common primary tumor of the liver and the fifth most common malignancy worldwide (112, 113). It is also the third most common cause of death related to cancer. Many of the predisposing conditions that are linked to the development of HCC involve chronic liver injury including hepatitis B and C, chronic alcohol abuse, primary sclerosing cholangitis, primary biliary cirrhosis, aflatoxin ingestion, and metabolic liver disease. In patients diagnosed with HCC, approximately 85% are considered unresectable at the time of diagnosis due to contraindications such as extrahepatic, bilobar or multi-focal disease, vascular invasion, or inadequate hepatic reserve due to underlying cirrhosis. Treatment options are mostly palliative and include intra-arterial chemotherapy, radiofrequency ablation, chemoembolization, ethanol injection, cryotherapy, or systemic chemotherapy (114-116). Systemic chemotherapy for HCC has been relatively unsuccessful with no effective regimen currently available. Thus, it is essential to identify and further understand the molecular mechanisms involved in the development of HCC to provide a basis for the development of more effective therapies.

Given that HCC typically appears in the setting of chronic injury, one hypothesis is that the environment of chronic injury paired with hepatocyte proliferation makes the hepatocytes

prone to genomic instability. For instance, in the cirrhotic liver, proper liver function is maintained by regenerating nodules made up of actively proliferating hepatocytes (117). At the same time, these hepatocytes are exposed to reactive oxygen species, toxic metabolites, and nutritional stress, all of which are genotoxic. Thus, the combination of ongoing cell proliferation and the exposure to genotoxic stress make for a more amenable environment for genetic alterations which induce transformation and cause some of these regenerative nodules to evolve into dysplastic nodules (118).

β-catenin activation is implicated in many different types of cancers and is likely one of the more important pathways to be aberrantly activated in HCC as identified in human and animal liver tumors (119-121). Abnormal localization of β-catenin in HCC was first shown by immunohistochemistry (122). Reports on the frequency of Ctnnb1 mutation in HCC have varied from study to study. One group reported that 25% of human HCC cases and 50% of murine HCCs in the setting of c-myc or H-ras over expression harbor mutations in the gene (123). Subsequent studies have reported Ctnnb1 mutation in a range from 12-34% of cases, while abnormal redistribution of β-catenin can be observed in up to 90% of cases (104, 124-127). While mutations in Ctnnb1 are often observed, β-catenin activation occurs in tumors due to a variety of other mechanisms as well. Some of the other mechanisms which have been reported are mutation in the *AXIN-1* and *AXIN-2* genes, frizzled-7 upregulation, and GSK3β inactivation (Fig. 4)(99, 128-130). Interestingly no study has examined the status of any of the Wnts in HCC.

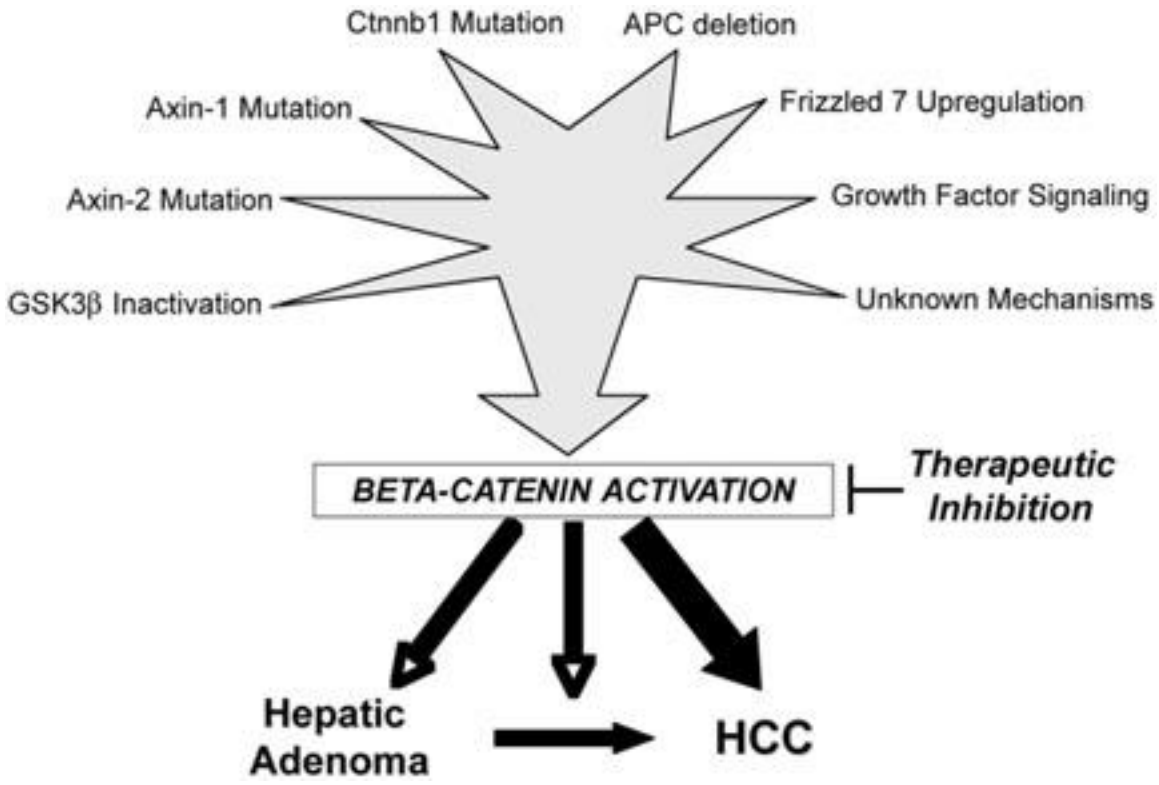


Figure 4: Mutations in Wnt/β-catenin pathway involved in hepatic adenoma and HCC.

In mice, liver specific deletion of APC induces β-catenin stabilization and increased HCC (131). Similarly, zebrafish heterozygous for APC also exhibit hepatic neoplasia, secondary to Wnt/β-catenin activation (132). However, mutations in APC are not commonly observed in HCC. Transgenic mouse models over expressing c-myc and/or TGF-β show mutation and/or nuclear translocation of β-catenin in liver tumors (133). Furthermore, β-catenin activation provides additional growth and invasive advantages in a model of liver cancer promotion with phenobarbital in c-myc/TGF-α transgenic mice. Intriguingly, no study to date has shown spontaneous HCC in transgenic mice over expressing wild-type or constitutively active β-catenin (89, 90, 134). Only simultaneous mutation of β–catenin and H-ras leads to 100% incidence of HCC in mice (135). These findings have clear implications in that β-catenin activation is likely a contributory factor in a significant subset of HCCs.

Hepatitis B and C, both major risk factors for the development of HCC, are linked to increases in β-catenin activity. In Hepatitis B virus (HBV) positive cases of HCC, hepatitis B virus encoded X antigen (HBxAg) is associated with decreased expression of E-cadherin and accumulation of β-catenin in the cytoplasm and/or nucleus and upregulation of the HBxAg effector URG11 leads to increased activation of β-catenin (136). This demonstrates multiple ways of regulation of β-catenin in HBV pathology. There have also been studies linking Wnt/β-catenin signaling to HCC that occurs in Hepatitis C virus (HCV) patients. Interestingly, the frequency of β-catenin gene mutations was around double in HCC occurring in HCV patients as compared to other causes (137). More than 40% of HCV associated HCCs demonstrate stabilizing mutations in Ctnnb1 with most occurring at Ser45. The mechanism of this predilection remains obscure calling for further investigation. An additional observation to this end has been an overall decrease in the incidence of HCC in HCV-positive patients, which have been on chemotherapy for their viral illness (138). While this might be due to decreased viral load in response to the antiviral agents, it is quite possible that these drugs have additional modulatory effects on signal transduction such as the Wnt/β-catenin pathway.

While it seems fairly clear that β-catenin activation has some role in the development of HCC, what it means for prognosis is yet unclear. Several studies have associated β-catenin activation with a poorer prognosis (126, 137). Likewise, larger tumor size was observed in HCC harboring mutations in Ctnnb1 compared to tumors which contained no mutation in β-catenin (119). On the other hand, pathway activation was linked to less aggressive tumors and a better prognosis in a couple other studies (139, 140). More comprehensive studies in the future will hopefully settle the discrepancies reported in the literature and find a definitive answer as to whether or not β-catenin activation predicts a better or worse outcome. It should be noted that in

a recent study from our lab, we identified activating mutations in exon-3 of CTNNB1 in 30% of HCCs and those tumors displayed greater tumor size with higher rates of microvascular invasion. However, CTNNB1-mutated tumors displayed less prominent cirrhosis suggesting that such mutation may lower the threshold for neoplasitic transformation such that HCC develops even without significant fibrosis. Another possibility is that β-catenin mutation might be an independent risk factor for HCC in addition to cirrhosis.

1.3.5 Targeting the Wnt pathway in the treatment of HCC

Given the role for β-catenin activation in HCC, there is significant interest in developing approaches for negatively regulating this pathway. Unfortunately, this pathway has proven difficult to target with drugs thus slowing the development of compounds which show a significant therapeutic benefit. Despite this fact, work in pre-clinical models is ongoing with several agents exhibiting clear potential. Several small molecule inhibitors are now in the early phases of drug discovery. Two fungal derivatives, PKF115-854 and CGP049090, are capable of blocking the interaction between β-catenin and TCF (141). These two compounds effectively decreased expression of two known targets of β-catenin, cyclin D1 and c-myc, as well as inhibited proliferation in HCT116 colon cancer cells. Another small molecule inhibitor, ICG-001, effectively prevents the interaction between β-catenin and the transcriptional co-activator CBP (142). This compound decreased expression of cyclin D1 and survivin, induced apoptosis specifically in colon cancer cells, and reduced the number of intestinal polyps by 42% in the APC^{min} mouse model.

In addition to small molecule inhibitors, several other factors have proven capable of negatively regulating the Wnt pathway. Cox-2 inhibitors can decrease levels of β-catenin and

diminish tumor size (143). Given the side effect profile associated with Cox-2 inhibitors, development of similar compounds which lack these side effects is underway. R-Etodolac is one such compound which lacks Cox-2 inhibitory activity but still effectively targets β-catenin in HCC cell lines (144). R-Etodolac also decreases HCC cell survival and proliferation suggesting this compound may have some therapeutic potential in the treatment of HCC. Another approach on the horizon is the development of antibodies directed at Wnt ligands. So far, antibodies against Wnt-1 and Wnt-2 have proven effective in decreasing β-catenin activity, as well as inhibiting cancer cell survival in vitro and tumor growth in vivo (145, 146). Although none of these therapies have reached clinical trials for the treatment of HCC or other tumors yet, further studies in pre-clinical models of HCC will hopefully make clinical trials a reality in the near future.

1.4 OVAL CELLS AND BILIARY DISEASE

1.4.1 Oval Cells/Atypical Ductular Proliferation

Although the resident parenchymal cells of the liver, namely the hepatocytes and biliary epithelial cells, display a highly efficient capacity for proliferation when needed, another cell type appears when the need for regeneration outstrips the capacity of these resident cells. This cell is often referred to as an adult liver progenitor cell or oval cell. While such cells are scarce in healthy liver, one proposed location is in the canal of Hering adjacent to the terminal ducts of the biliary tree (147). However, three other potential progenitor cell niches have been reported including intralobular bile ducts, peri-ductal mononuclear cells, and peribiliary hepatocytes

(148). Given that oval cells express markers specific to hepatocytes and to cholangiocytes many believe that these cells are bipotential in nature (149, 150). Indeed, in vitro and in vivo studies have reported the ability of oval cells to differentiate into either epithelial cell type (151-153). Several stem cell markers (CD34, flt3, LIF, and c-kit) are also expressed during oval cell activation suggesting a potential stem cell-like behavior for oval cells (154-157). It has also been suggested that these cells may come from a hematopoietic origin as expression of sca-1 and thy-1 is observed in a subpopulation of oval cells in rodent liver (158, 159). Although, several studies have refuted the possibility of significant hematopoietic involvement in the oval cell response or showed it to be a result of fusion (160, 161).

Generally, the oval cell population is quiescent and only proliferates at times when the hepatocytes and cholangiocytes themselves are impaired or overwhelmed. Not surprisingly oval cell activation is observed in conditions associated with chronic liver injury and development of hepatocellular carcinoma (HCC) such as chronic viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (150, 162). Greater than 50% of human HCCs express one or more markers of progenitor cells or oncofetal proteins such as α-fetoprotein, cytokeratins 7, 14 or 19 (163-165). This is even more relevant since around half of the small cell dysplastic foci, the earliest cancer precursor lesions known to date, consist of progenitor cells and their progeny, suggesting that these lesions are the result of activation and proliferation of progenitor cells (166). Another report has shown that half of hepatocellular adenomas consist of progenitor cells and intermediate hepatocytes (167). Given such association with liver cancer, great interest lies in identification of the molecular characteristics of this cell population given that these cells may be potential targets for therapy. One thing that is clear is that the progenitor population is heterogeneous in nature and may represent several different cellular populations.

1.4.2 DDC Diet Model

The molecular mechanisms involved in the induction and propagation of the oval cell response are currently not well understood. In an attempt to decipher the molecular mechanisms involved, multiple animal models have been developed for activation of oval cells (168-174). The primary model used in rat is the 2-acetylaminofluorine/partial hepatectomy (2-AAF/PH) model (174). The benefit of this model is that 2-AAF blocks hepatocyte proliferation facilitating a need for another cell population to execute liver regeneration after partial hepatectomy. Thus, oval cells appear in the periportal region and successfully regenerate the lost hepatic mass. While the 2-AAF/PH model is useful for inducing oval cell activation, genetic manipulation in the rat is difficult and expensive. Therefore, the development of mouse models of oval cell activation is essential for studying the molecular mechanisms of this response.

One model commonly used in mice is the 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet model. This model has been used for studying Mallory body formation for about 30 years, but only gained interest as an oval cell model in the last decade (171, 175). The response in liver after feeding mice the DDC diet is often described as atypical ductular proliferation (ADP) (171). Histological examination of the periportal area following administration of DDC for 1-4 weeks reveals significant proliferation of the cholangiocytes, formation of atypical ductules, periportal inflammation, and the development of porphyrin plugs clogging the bile ducts. Clogging of the bile ducts induces biliary stasis and a subsequent rise in serum bilirubin levels indicative of intrahepatic cholestasis. It is believed that hepatic oval cells arise as a response to hepatic injury in this model. A more recent study presented the DDC diet protocol as a model of xenobiotic-induced cholangiopathy representative of sclerosing cholangitis and biliary cirrhosis (176). This group found that DDC feeding led to bile duct injury which induced

reactive cholangiocytes and was associated with pericholangitis and periductal fibrosis. Eventually, periductal fibrosis progressed to portal-portal bridging fibrosis and segmental bile duct obstruction occurred. Given that the DDC diet protocol replicates many of the features of chronic cholangiopathies; it is a valuable model for the investigation of the mechanism of biliary disease and the subsequent reparative process.

Preliminary evidence indicates that the Wnt pathway may be involved in the ADP response that occurs after exposure to DDC. We found that significant colocalization of the ductular marker A6 and β-catenin occurred after DDC feeding in mice (Figure 5, (177)). Ablation of β-catenin in the liver, utilizing the albumin-driven cre recombinase model, caused a blunted ADP indicating that β-catenin may have some role in the induction or proliferation of ductular cells during this response to DDC. Another group has likewise reported expression of β-catenin in ADP as well as an induction of multiple Wnt ligands after DDC feeding (178). More extensive studies are essential to elucidate what role β-catenin is playing during the response to DDC feeding and by association what potential role it may play in ADP and biliary disease.

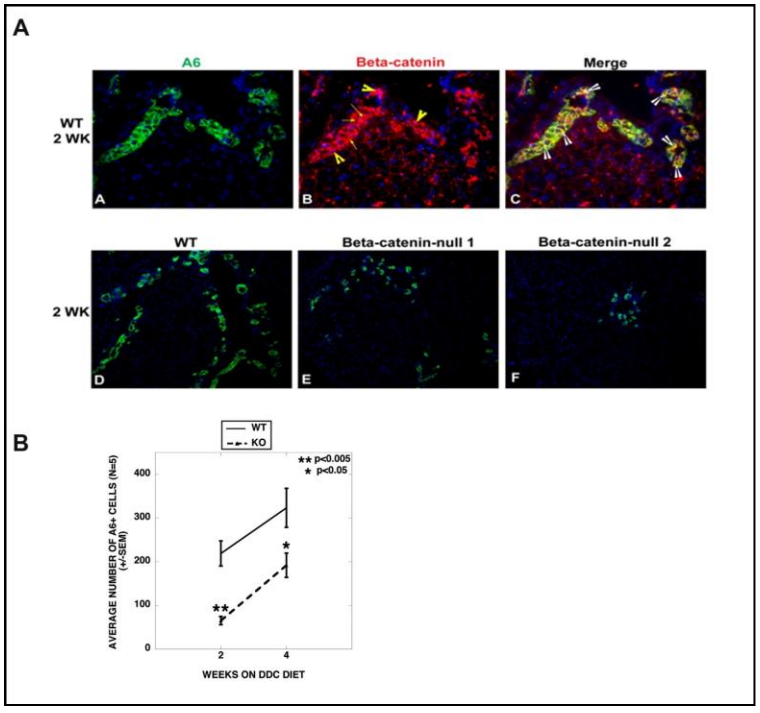


Figure 5: CTNNB1 KO mice exhibit a decrease in ADP following DDC feeding. **(A)** A6 (green) and β-catenin (red) colocalize (yellow) in ADP of WT mice after 2 weeks of DDC feeding. The number of A6 positive cells in KO liver after 2 weeks of DDC is less than that observed in WT liver. **(B)** Quantification of A6 positive cells in WT and KO liver after 2 and 4 weeks of DDC feeding.

1.4.3 Cholestatic Liver Disease

The liver plays a primary role in the uptake, metabolism, and excretion of bile acids. The hepatocytes metabolize and secrete bile acids into the canaliculus where it begins its journey through the bile ducts which make up the biliary tree. When the biliary tree is damaged by inflammation or toxins, intrahepatic bile flow is compromised leading to cholestasis. Several diseases have been associated with cholestasis including those that affect the pediatric population (biliary atresia and progressive familial intrahepatic cholestasis (PFIC)) and those that impact the adult population (primary sclerosing cholangitis (PSC) and primary biliary cirrhosis (PBC)) (reviewed in (179)). Of the cholestatic diseases, PSC and PBC are particularly associated with chronic injury/damage to the bile ducts which can lead to liver cirrhosis, failure, and cancer.

Although PSC is not a relatively common disease, it has become one of the most common indications for liver transplantation in the adult population. PSC occurs more often in men and is frequently observed in patients who have inflammatory bowel disease (180, 181). The disease is characterized histologically by the damage and loss of both medium and large-sized bile ducts that eventually affects the smaller ducts due to biliary obstruction (182). Concentric periductal fibrosis, also known as "onion-skinning", is a pathological feature commonly observed in PSC which ultimately develops into a bile duct scar. The etiology of PSC is poorly defined with the understanding of the molecular mechanisms of disease currently unknown; though, the current body of evidence points to immune mechanisms as likely involved in the pathogenesis. One current hypothesis relates to autoimmune mechanisms involving exposure of a genetically predisposed individual to an insult to the bile duct, such as a viral infection, which leads to immune recognition of the patient's bile ducts as foreign and an ensuing

autoimmune response against the biliary tree. Interestingly, anti-neutrophil cytoplasmic antibodies are found in the sera of up to 88% of PSC patients (183).

Much like PSC, the molecular pathogenesis of PBC is not very well-defined. PBC is also likely to involve immune mechanisms and is characterized histologically by destruction of biliary epithelial cells lining the small bile ducts and significant infiltration of inflammatory cells around the portal tract (184). Autoantibodies to pyruvate dehydrogenase were identified in the sera of PBC patients and linked to the development of the disease, thus implicating autoimmunity in the pathogenesis (185). However, autoimmunity does not seem to explain the entire pathological process and treatments directed at autoreactive T-cells have not been as successful as one would expect for an autoimmune disease. Other mechanisms of BEC loss have been explored including epithelial to mesenchymal transition (EMT) and cellular senescence (186, 187). Although the molecular mechanisms involved in the pathology of PBC are not well understood, one study performed gene array analysis to examine what pathways are affected in PBC (188). Interestingly, the analysis performed in this study implicated the Wnt pathway in that expression of β-catenin, several Wnt ligands, and several downstream targets of canonical Wnt signaling were all upregulated. It is possible that β-catenin activity may have some role in the development of cirrhosis and/or cancer in these patients. On the other hand, β-catenin may also play some role in the liver's attempt to repair and regenerate from the injury. Given that liver transplantation is the only curative treatment for both PSC and PBC patients, exploration of pathways such as the Wnt pathway as potential targets for new therapies is warranted.

1.4.4 Hepatocellular carcinoma in PSC and PBC

Given that many of the chronic cholangiopathies share features of chronic injury, inflammation, and the development of fibrosis and cirrhosis, it is reasonable to expect that the development of cancer could occur as a long-term consequence of these diseases. In the case of PSC, cholangiocarcinoma is the more commonly observed cancer, although cases of HCC associated with PSC have been noted. The overall frequency of hepatobiliary malignancy, including HCC, cholangiocarcinoma, and gallbladder cancer, was reported at 13.3% (189). Another study which examined PSC patients receiving liver transplantation found that 2% of the patients had HCC at the time of transplant (190). Although the increase in incidence is small in this population, it appears that there is some predisposition for developing HCC. It is likely that patients with severely advanced disease are more prone. Future studies encompassing larger patient populations will hopefully address whether advanced disease is further predictive of developing HCC.

The occurrence of HCC seems to be a little more common in patients with PBC. While the overall incidence is around 3% in the PBC population, the incidence is over 11% with more advanced (stage III or IV) PBC (191). The incidence observed in late stage PBC was similar to that observed in HCV-related cirrhosis suggesting that cirrhosis seems to be the primary factor involved here. A more comprehensive study was performed recently in Europe which included over 700 PBC patients and found very similar results (192). This study also found that survival was lower in patients with PBC-associated HCC when compared to patients with HCV-associated HCC if you exclude patients who received a liver transplant. The difference in survival might be explained by the fact that a majority of the patients in the PBC study group who developed HCC had significantly advanced disease (Stage IV). Thus, the data further

reiterate that advanced cirrhosis in primary biliary cirrhosis, much like other diseases of the liver, is an important risk factor for the development of HCC. One management strategy might involve careful monitoring of these patients which could identify high risk patients prior to development of advanced disease and hopefully allow aggressive treatment to prevent the development of HCC.

2.0 MATERIALS AND METHODS

2.1 ANIMAL MODELS

2.1.1 Mutagenesis and creation of β-catenin transgenic mouse

Using the GeneTailor^{TM} site-directed mutagenesis kit, Serine-45 in *CTNNB1* was mutated to aspartic acid (S45D), alanine (S45A), and phenylalanine (S45F), which were previously shown to prevent ubiquitination of β-catenin (8). These constructs, along with *CTNNB1* exon-3 deletion mutant (Δ-*nt:282-630)*, were tested for β-catenin activity by Topflash assay (described below) in HEK293 cells (193). cDNA containing S45D mutation was inserted into *Bam*HI sites of an albumin promoter/enhancer-driven expression vector to generate transgenic mice in FVB background. Two independent founder lines were identified and based on comparable β-catenin expression only one line was expanded for analysis. The transgenic line was maintained as homozygous and henceforth referred to as TG mice. Only male mice were used for all experiments and age-matched wild-type FVB mice (WT) served as controls. At sacrifice, portions of 3 different lobes from excised liver were fixed in 10% neutral buffered formalin and processed for paraffin embedding. A portion of liver was frozen in the Tissue-Tek OTC compound for frozen sections. The remaining liver was snap frozen in liquid nitrogen and stored

at -80°C. All animal studies were performed in strict accordance with Institutional and NIH guidelines.

2.1.2 Creation of KO mouse

Homozygous floxed β-catenin mice (C57BL/6 strain) and albumin-Cre mice (C57BL/6 strain) were obtained from Jackson Laboratories (Bar Harbor, ME). Homozygous floxed β-catenin mice were bred to albumin-Cre mice and the offspring carrying a floxed β-catenin allele and albumin-Cre were again bred to the homozygous floxed β-catenin mice. This led to a floxed allele and a floxdel-deleted (floxdel) allele of Ctnnb1 and are referred to as *Ctnnb1*$^{loxp/loxp}$; *Alb-Cre*$^{+/-}$ or knockout (KO) mice throughout. For all experimentation and analysis, the following genotypes were used as controls unless mentioned otherwise: Ctnnb1$^{loxp/loxp}$; Alb-Cre$^{-/-}$ or Ctnnb1$^{loxp/Wt}$; Alb-Cre$^{+/-}$ or Ctnnb1$^{loxp/Wt}$; Alb-Cre$^{-/-}$, and are referred to as wild-type (WT) throughout. Only male mice were used for all experiments. At the time of sacrifice, retro-orbital bleed was performed to isolate serum for serum biochemical analysis. Portions of 3 different lobes from excised liver were fixed in 10% neutral buffered formalin and processed for paraffin embedding. A portion of liver was frozen in the Tissue-Tek OTC compound for frozen sections. The remaining liver was snap frozen in liquid nitrogen and stored at -80°C. All animal studies were performed in strict accordance with the Institutional Animal Use and Care Committee at the University of Pittsburgh School of Medicine and NIH guidelines.

2.1.3 DEN-induced carcinogenesis

At 15 days-old, TG and WT mice were administered an intraperitoneal (IP) injection of DEN (5mg/kg) and sacrificed at 6 or 9 months. Livers were processed for histology, and lesions characterized as dysplastic foci, hepatic adenoma, or HCC, based on tumor size, thickness of hepatic plate and presence of mitotic figures under the supervision of a pathologist (T.W.).

2.1.4 DEN-induction and Peg-IFN treatment

At 15 days of age, TG mice were given a single intraperitoneal (IP) injection of diethylnitrosamine (DEN) (Sigma) at a dose of 5 mg/kg of body weight as described above. At 2.5 months following DEN exposure, mice received subcutaneous injection of 0.9% saline or Peg-IFN (n=4). Mice received 7000U or 70,000U once weekly for six weeks after which they were sacrificed. Samples from multiple lobes of the liver were fixed in formalin and embedded in paraffin. The remaining liver tissue was snap frozen and stored at -80ºC for later analysis. All animal studies were performed in strict accordance with the institutional animal use and care committee at the University of Pittsburgh School of Medicine and Institutes of Health guidelines.

2.1.5 DDC diet feeding

Mice were fed a special diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC, Bioserve, Frenchtown, NJ) for periods of time ranging from 3 to 200 days to induce atypical ductular proliferation, which has been described previously (171). For recovery study, mice were

fed DDC diet for 28 days, followed by reinstitution of normal chow for 2 days at which time animals were sacrificed.

2.1.6 Serum biochemistry

Serum biochemical measurements were performed by the University of Pittsburgh Department of Pathology Lab Support Services. Total bilirubin, alkaline phosphatase, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were measured on samples taken prior to sacrifice at multiple time points.

2.2 CELL CULTURE

2.2.1 Cell lines and reagents

Human HCC cell lines, Hep3B, HepG2, Huh-7, and Snu-449, were obtained from American Tissue Type Culture (Manassas, VA). Cells were cultured in Eagle's minimum essential medium (EMEM, Cambrex, Walkersville, MD) supplemented with either 2 or 10% fetal bovine serum and incubated in 5% CO2 at 37ºC.

2.2.2 Drug treatment

Treatments were begun when cells were approximately 30-50% confluent. Peg-IFN was utilized at a concentration of 100 U/ml. Ribavirin was utilized at a concentration of 5, 50, or 200 µM.

Cells were treated and media changed every 48 hr for 1-4 doses, at which time the cells were harvested for analysis.

2.2.3 β-catenin/Tcf Transcription Reporter Assay

HepG2 cells were plated in six-well plates and transiently transfected with the plasmids TOPflash and FOPflash (Upstate Biotechnology, Lake Placid, NY). TOPflash has three copies of the Tcf/Lef sites upstream of a thymidine kinase (TK) promoter and the firefly luciferase gene. FOPflash contains mutated Tcf/Lef sites and is used as control for measuring nonspecific activation of the reporter. All transfections were performed with FuGene HD reagent (Roche, Indianapolis, IN) and 0.8 μg of TOPFlash or FOPFlash plasmids. To normalize transfection efficiency, cells were co-transfected with 0.1 μg of the internal control reporter *Renilla reniformis* luciferase driven under the TK promoter (pRL-TK; Promega, Madison, WI). Luciferase assay was performed using the Dual Luciferase Assay System kit according to the manufacturer's protocols (Promega). Relative luciferase activity was reported as a ratio of firefly/renilla luciferase activity. Experiments were performed in triplicate.

2.2.4 SiRNA transfection

HepG2 cells were cultured in 6-well plates in 2 ml of EMEM medium complemented with 10% FBS and treated at 30-50% of confluency by adding two doses of Peg-IFN (100 U/ml) followed by serum starvation for 16 hours and then transfected by adding pre-validated human RanBP3 siRNA (Ambion, Inc, Cat No. 4392420) or control (scrambled) siRNA (Ambion, Inc, Cat No. 4390846) at a final concentration of 10 nM using the Lipofectamine reagent (Invitrogen,

Carlsbad, CA). Cells were rinsed with the medium after 24 hours of incubation and then transfected with TOPFlash or FOPFlash reporter plasmid in presence of FuGene HD reagent (Roche, Indianapolis, IN) and incubated for 24 hours before an additional dose of 10 nM RanBP3 siRNA or the control (scrambled) siRNA was added. Luciferase activity was measured 48 hours after transfection. Experiments were performed in triplicate and repeated at least 2 times.

2.3 OTHER PROCEDURES

2.3.1 Protein extraction and western blot analysis

Whole cell lysates were extracted in radioimmunoprecipitation assay (RIPA) buffer with protease inhibitor and phosphatase inhibitor cocktail 1 and 2 (Sigma, inhibitors at a dilution of 1:100). Nuclear extraction was performed using NE-PER nuclear and cytoplasmic extraction reagents according to the manufacturer's protocol (Pierce). The concentration of total protein in the lysates was determined by bicinchoninic acid protein assay with bovine serum albumin used as a standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed with 20-100 μg of protein that was resolved on Bio-Rad precast gels (7.5% or 4-15% gradient gels) under reducing conditions by using the Mini-Protean electrophoresis module assembly (Bio-Rad, Hercules, CA). This was followed by a 1 hour transfer at constant voltage (100V) in transfer buffer (25 mmol/L Tris [pH 8.3], 192 mmol/L glycine, 20% methanol, and 0.025% sodium dodecyl sulfate) to polyvinylidene difluoride membranes (PVDF, Millipore, Bedford, MA) by using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad).

For western blot analysis, membranes were first blocked with 5% milk (if rabbit or mouse primary antibody used) or 5% BSA (if goat antibody used) for 30-45 min. at room temperature or overnight at 4ºC. Membranes were then incubated with primary antibody in 5% milk or BSA for 1 hour at RT followed by 2 washes in 1% milk or BSA. Primary antibodies used are listed in Table 1. Membranes were then incubated with appropriate HRP-conjugated secondary antibody (Chemicon International Inc., Temecula, CA) at concentrations of 1:10,000 to 1:50,000 in 1% milk or BSA for 1 hour at RT followed by 2 washes with blotto. Blots were visualized with Western Lightning™ chemiluminescence kit (PerkinElmer Life Sciences, Boston, MA). Autoradiographs were scanned and analyzed for densitometry using the ImageJ software.

Table 1: Primary antibodies used for western blotting

Antibody Target	**Concentration**	**Source**
β-catenin	1:1,000	BD Biosciences
E-cadherin	1:500	BD Biosciences
Met	1:200	Santa Cruz
Glutamine Synthetase	1:200	Santa Cruz
Cyclin D1	1:500	Neomarkers
RanBP3	1:1,000	Abcam
GAPDH	1:1,000	Santa Cruz
Actin	1:5,000	Chemicon
Lamin B1	1:200-300	Santa Cruz

2.3.2 Real-time PCR

mRNA was isolated using Trizol and cDNA created from cell lines as previously described. Real-time PCR was run for Dkk-1 (sense: 5'-ctc ggt tct caa ttc caa cg-3'; antisense: 5'-gca ctc ctc gtc ctc tg-3') and RanBP3 (sense: 5'-tga aga gga agc ctg tga gaa-3'; antisense: 5'-gtg tcc agc att ctc cat gtc-3') on ABI Prism 7000 sequence detection system (Applied biosystems). Three samples were pooled for each condition and run in duplicate. Treated samples were compared to control using the ΔΔCt method and plotted as fold change. Actin was used as a control.

2.3.3 Histology, immunohistochemistry, and special stains

Tissues fixed in 10% formalin were embedded in paraffin and 4 μm sections cut onto Superfrost Plus glass slides (Fisher Scientific, Pittsburgh, PA) were used for H&E staining or immunohistochemistry. For H&E staining sections were rehydrated by passing through xylene, graded alcohol, and distilled water. Slides were then placed in Shandon's hematoxylin followed by washes in dH2O and then placed in eosin. Slides were then passed through increasing grades of alcohol, followed by xylene and then coverslipped. For immunohistochemistry, sections were rehydrated by passing through xylene, graded alcohol, and distilled water. Antigen retrieval was performed and endogenous peroxide was inactivated using 3% hydrogen peroxide (Sigma). Blocking was performed using Superblock for 10 minutes and then sections were incubated with primary antibody for 1 hour at room temperature. Primary antibodies used are listed in Table 2. Sections were then washed with PBS and incubated with appropriate biotin-conjugated secondary antibody for 30 minutes at room temperature. Sections were washed with PBS and then incubated with ABC reagent for 30 minutes at room temperature. Wash with PBS and then

incubate with DAB for 1-5 minutes. Sections were counterstained with Shandon hematoxylin solution (Sigma) and cover slipped using Cytoseal XYL (Richard Allen Scientific, Kalamazoo, MI). For negative control, the sections were incubated with secondary antibodies only. Slides were viewed under an Axioskop 40 (Zeiss) upright research microscope and digital images were obtained. Collages were prepared using Adobe Photoshop CS3 software (Adobe, San Jose, CA). For quantification of Ki-67, PCNA and CD45 positive cells, 5 high power field images were taken for each animal and counted utilizing Zeiss Axiovision software. All counts were averaged among each group (TG or WT) at each time point.

For measurement of fibrosis, trichrome staining was performed by the Department of Pathology Histology Services. Photomicrographs were taken at 50x magnification and % area of fibrosis measured using Adobe photoshop as previously described.

Table 2: Primary antibodies used for immunohistochemistry

Antibody Target	**Concentration**	**Antigen Retrieval**	**Source**
β-catenin	1:50	Citrate Buffer	Santa Cruz
Cyclin D1	1:50	Citrate Buffer	Neomarkers
Glutamine Synthetase	1:50	Citrate Buffer	Santa Cruz
Ki-67	1:50	Dako A.R. Buffer	Dako
PCNA	1:4000	Zinc Sulfate	Santa Cruz
CK19	1:100	Citrate Buffer	Santa Cruz
CD45	1:100	Citrate Buffer	Santa Cruz
α-fetoprotein	1:50	Citrate Buffer	Santa Cruz
HNF1β	1:50	Citrate Buffer	Santa Cruz
Trop-2	1:7	Citrate Buffer	R&D Systems

2.3.4 Immunofluorescence

Frozen sections were fixed with ice cold acetone for 10 minutes, washed in phosphate-buffered saline (PBS), and incubated with 10% normal serum (type dependent on source of secondary antibody) prepared in 1% bovine serum albumin (BSA) in PBS for 20 minutes to suppress non-specific IgG binding. Primary antibody was prepared in 1% BSA in PBS and applied to sections for 1 hour at room temperature. Primary antibodies used are listed in Table 3. Following washing with PBS, sections were incubated in the dark with appropriate fluorochrome-conjugated secondary antibody in 1% BSA in PBS for 1 hour. Following washing with PBS, sections were counterstained and mounted using mounting medium containing 4′6-diamidino-2-phenylindole (DAPI). Fluorescent microscopy was performed on a Zeiss Axioscope microscope. For quantification of A6 positive cells, 5 high power field images were taken around a periportal region for each animal and counted utilizing Zeiss Axiovision software. All counts were averaged among each group (TG, KO, or WT) at each time point.

Table 3: Primary antibodies used for immunofluorescence

Antibody Target	**Concentration**	**Source**
A6	1:100	Gift from Dr. Valentina Factor
β-catenin	1:50	Santa Cruz

2.3.5 Statistical analysis

All experiments were performed three or more times or with three or more animals and representative data are presented. Quantification of positive cells (A6, PCNA, CD45, Ki-67), serum biochemistry measurements, Topflash assays, and optical density measurements were

compared for statistical analysis by Student *T* test (Excel) and *P* value of less than 0.05 or 0.01 was considered significant or extremely significant, respectively.

3.0 SERINE-45 MUTATED BETA-CATENIN IN NORMAL AND DYSPLASTIC LIVER GROWTH

3.1 ABSTRACT

Hepatocellular carcinoma (HCC) is a disease of poor prognosis with limited therapeutic options due to limited knowledge of the molecular mechanisms of this disease. Despite this, mutations affecting phosphorylation sites on β-catenin are common, with serine 45 being the most commonly mutated single site. We have developed a transgenic mouse that expresses Ser45 mutated β-catenin (TG) in hepatocytes to study the effects on normal and dysplastic liver growth. Increased LW/BW of 10-20% was observed as early as 15 days postnatally in TG. Increased levels of nuclear β-catenin were observed at 1 month, which coincided with increased cyclin D1 and Ki-67-positive hepatocytes compared to WT. Interestingly, elevated β-catenin was observed at the hepatocyte membrane at 2 months with no ongoing proliferation and a return to normal LW/BW. At 2 months, association of β-catenin with E-cadherin and Met is increased in TG liver. β-Catenin over expressing TG mice, when followed up to 12 months showed no signs of spontaneous tumorigenesis. However, intra-peritoneal delivery of a single dose of diethylnitrosamine (DEN) a known hepato-carcinogen at day 15 after birth, induced HCC at 6 months in TG mice only. Tumors in TG livers showed upregulation of β-catenin, cyclin-D1 and unique genetic aberrations while other canonical targets were unremarkable. In conclusion, β-

catenin over expression offers a modest growth advantage post-natally which is regulated by membranous sequestration of the protein. While expression of β-catenin transgene in hepatocytes was insufficient to induce spontaneous liver tumors, it led to increased susceptibility to DEN-induced HCC.

3.2 BACKGROUND

Wnt/β-catenin signaling is evolutionarily well-conserved pathway and important in liver health and repair (194). In adult liver, β-catenin signaling is essentially quiescent with active-β-catenin restricted to hepatocytes in centrizonal area where it regulates expression of genes such as glutamine synthetase (*GS*) and others involved in xenobiotic metabolism (195). In other hepatocytes, β-catenin steady state is achieved by phosphorylation at key serine/threonine residues and subsequent degradation, and is predominantly localized to membrane to mediate cell-cell adhesion by forming a bridge between E-cadherin and actin cytoskeleton (196).

Activation of β-catenin signaling during liver regeneration has been reported in rats and mice (84, 91, 92, 95). While a positive regulator in the activity of normal liver growth, aberrant activation of the Wnt/β-catenin pathway is implicated in hepatocarcinogenesis, although exact mechanism remains elusive. A significant subset of hepatocellular cancers (HCC) and hepatoblastomas display Wnt pathway activation due to various mechanisms including mutations in *CTNNB1*, the gene encoding for β-catenin (100, 123). Missense point mutations observed in tumors affect key phosphorylation sites involved in β-catenin degradation, with serine-45 being commonly altered (120). While no current animal study has shown increased tumorigenesis using single site mutations in this gene, liver targeted deletion of APC, another negative

regulator, induces β-catenin stabilization and increased incidence of spontaneous HCC (131). Similarly, zebrafish heterozygous for APC also exhibit hepatic neoplasia, secondary to Wnt/β-catenin activation (132). Interestingly, simultaneous mutation of β–catenin and H-ras leads to 100% incidence of HCC in mice (135). Regardless of the mechanism, it is clear multiple aberrations converge at β-catenin activation likely making it an initiating or contributory factor in a significant subset of HCCs and thus an attractive therapeutic target. Given that single site mutations are commonly observed in HCC, we sought to over express serine-45-mutated *CTNNB1* in the liver to characterize these animals for hepatic growth.

To address the role of a specific mutation in hepatic tumorigenesis, we developed a transgenic mouse model over expressing Ser45 mutated β-catenin under albumin promoter/enhancer (TG). In this study, we use this model to examine the effect of Ser45 mutated β-catenin on normal post-natal liver growth and both spontaneous and diethylnitrosamine (DEN)-induced hepatocarcinogenesis. We hypothesized that this mutation will provide a significant growth advantage to the hepatocyte in normal growth conditions, as well as ultimately initiate and/or promote dysplastic growth in the mouse liver.

3.3 RESULTS

3.3.1 Generation of Serine-45 mutant β-catenin TG mice

Previously, it was shown that mutation of the four residues (Serine-33, 37, 45 and Threonine-41) to either alanine (A) or aspartic acid (D) resulted in decreased ubiquitin tagging of β-catenin (8). S45A, S45D, S45F or active-β-catenin mutant (Δ-*nt:282-630),* were transfected in HEK293 cells

along with TOPflash or FOPflash reporters. Transcriptional activity in response to the deletion mutant was increased 25-fold above empty vector alone (p=0.000435), while all other mutants did not enhance reporter activity (Fig. 6a). Despite these observations, and based on reported point mutations affecting this site in HCC, we generated liver-specific TG mice by placing S45D-β-catenin gene under the transcriptional control of albumin promoter/enhancer as discussed in methods (Fig. 6b).

3.3.2 Transient in vivo liver growth advantage in TG

Age-matched TG and WT mice were compared for liver weight and liver weight/body weight (p=0.02) as compared to WT (Fig. 6c). Concomitant increases in LW/BW were also observed at 15D (p= 0.035) and 30D (p=0.0004) in TG mice. Interestingly, no significant differences in these parameters were evident in 2 months or older WT and TG mice.

Significant increase in basal cell proliferation was evident in 1M-old TG livers (p<0.0001) as assessed by Ki-67 IHC (Fig. 6d). WT and TG mice both show high proliferation at 15D, as postnatal hepatic growth is normally rampant and hence may be masking transgene–induced cell proliferation (Fig. 6e). Comparable cell proliferation was observed in 2M and older WT and TG livers.

To examine basis of enhanced cell proliferation, we compared the expression of cyclin-D1 in livers from 1M- and 2M- old animals. Increased levels of cyclin-D1 were evident in TG livers at 1M only (Fig. 6f). GS, another target of β-catenin, was unchanged in TG and WT livers at either time (Fig. 6e).

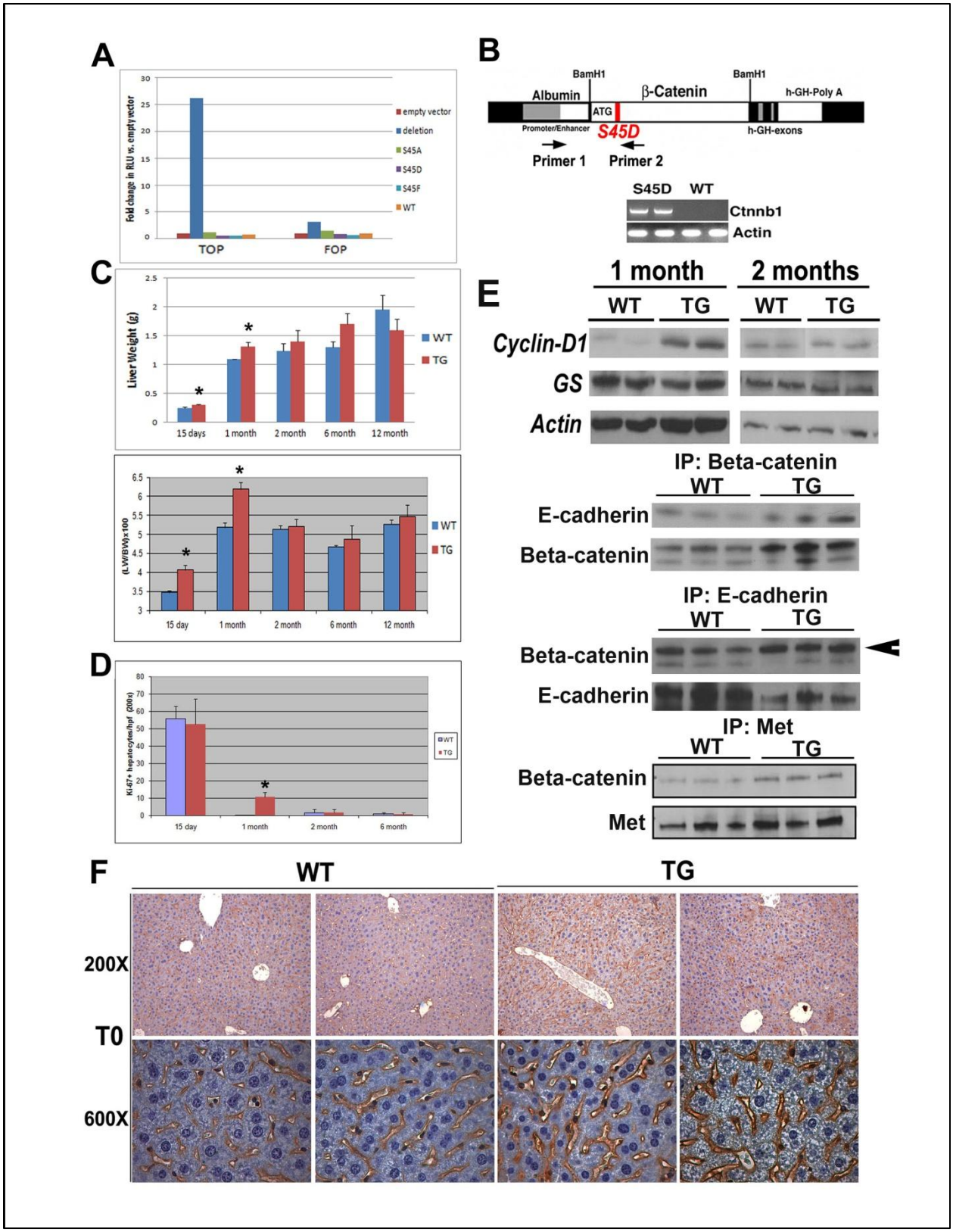


Figure 6: Creation and characterization of TG mice.

(A) Topflash assay showing transcriptional response in HEK 293 cells following transfection with various β-catenin mutant expression vectors. **(B)** Full length human *Ctnnb1* containing a point mutation (S45D) was inserted downstream of an albumin promoter/enhancer and injected into FVB mouse eggs to create transgenic mice. Genomic DNA was subjected to PCR using indicated primers (X and Y) whose position is indicated. Representative PCR shows the presence of transgene in TG mice and not WT mice. **(C)** Liver weight in TG mice and age-matched WT (n≥3) (upper panel). Liver weight to body weight ratio (LW/BW) in TG and age-matched WT (n≥3) (lower panel). **(D)** Increase in the number of Ki-67 positive hepatocytes in TG liver at 1 month compared to WT. **(E)** Western blot showing expression levels of total cyclin D1 and GS in TG and WT liver at 1 and 2 months of age. An increase in cyclin D1 is observed in TG at 1 month. Actin was used as a loading control. IP studies from three representative livers shows enhanced association of β-catenin with E-cadherin and β-catenin with Met in adult TG livers as compared to WT. **(F)** Immunohistochemistry for β-catenin reveals excess membranous localization in TG.

Since cyclin-D1 was not elevated at 2M, we hypothesized that excess β-catenin in TG livers might be associating with its membrane partners. Indeed, association of β-catenin with E-cadherin and Met by co-precipitation was greater in 2M or older TG livers (Fig. 6e). Similarly, while it was more intense in TG, β-catenin localized to the hepatocyte membrane in both WT and TG at 2M (Fig. 6f).

Taken together, the above findings indicate that TG mice show temporal β-catenin activation during early postnatal development, followed by adaptive changes other than ubiquitin-mediated degradation, which prevent excessive β-catenin activation through its membranous sequestration.

3.3.3 Accelerated hepatic tumorigenesis in TG mice following exposure to DEN

Age matched TG and WT mice were followed up to 12 months, but no spontaneous tumors were evident, despite the presence of mutant-β-catenin in TG livers. We next investigated the susceptibility of TG and WT mice to DEN-induced carcinogenesis as described in methods. We observed multiple liver tumors in the form of dysplastic foci, hepatic adenomas and HCC in TG mice at 6M and 9M and in WT at 9M after DEN exposure (Table 4). The preponderance and stage of tumors was clearly higher in TG livers. While only 2 out of 8 WT mice exhibited tumors with histological attributes of HCC, 70% OF TG mice showed HCC at 9 months (Table 4). Thus, over expression of β-catenin accelerates tumorigenesis and progression to HCC following DEN exposure.

Table 4: Hepatic tumors in WT and TG mice at 6 and 9 months after DEN exposure

	WT	**TG**
6 months post-DEN		
Tumor Incidence	0/8 (0%)	5/5 (100%)
Dysplastic Foci	0/8 (0%)	5/5 (100%)
Hepatic Adenoma	0/8 (0%)	2/5 (40%)
HCC	0/8 (0%)	3/5 (60%)
9 months post-DEN		
Tumor Incidence	4/8 (50%)	6/7 (86%)
Dysplastic Foci	3/8 (38%)	4/7 (57%)
Hepatic Adenoma	4/8 (50%)	6/7 (86%)
HCC	2/8 (25%)	5/7 (71%)

3.3.4 Tumors in TG show β-catenin activation

To examine state of Wnt signaling in TG liver tumors, we performed IHC for β-catenin and downstream targets of the pathway. Cytoplasmic and nuclear localization of β-catenin was observed in tumors in TG mice at 6 and 9 months after DEN exposure (Fig. 7a and 8a). This coincided with increased cyclin-D1 in TG tumors (Fig. 7b and 8b). Interestingly, GS was not dramatically different in the two groups at anytime. TG tumors were also notably PCNA-positive (Fig. 7b and 8b). As mentioned previously, WT livers show no tumors at 6 months after DEN exposure but at 9 months 50% of WT bear hepatic tumors (Table 4). Most tumors observed exhibited membranous β-catenin and a few cyclin-D1-positive cells (Fig. 8a and 8b). However a minority of tumors in WT does exhibit nuclear/cytoplasmic β-catenin and many cyclin-D1- and PCNA-positive cells, although GS was unremarkable (Fig. 8c). Thus, at 9 months following DEN exposure, TG mice continue to show predominant hepatic tumors with β-catenin activation, while WT livers exhibit admix of β-catenin-active and inactive tumors.

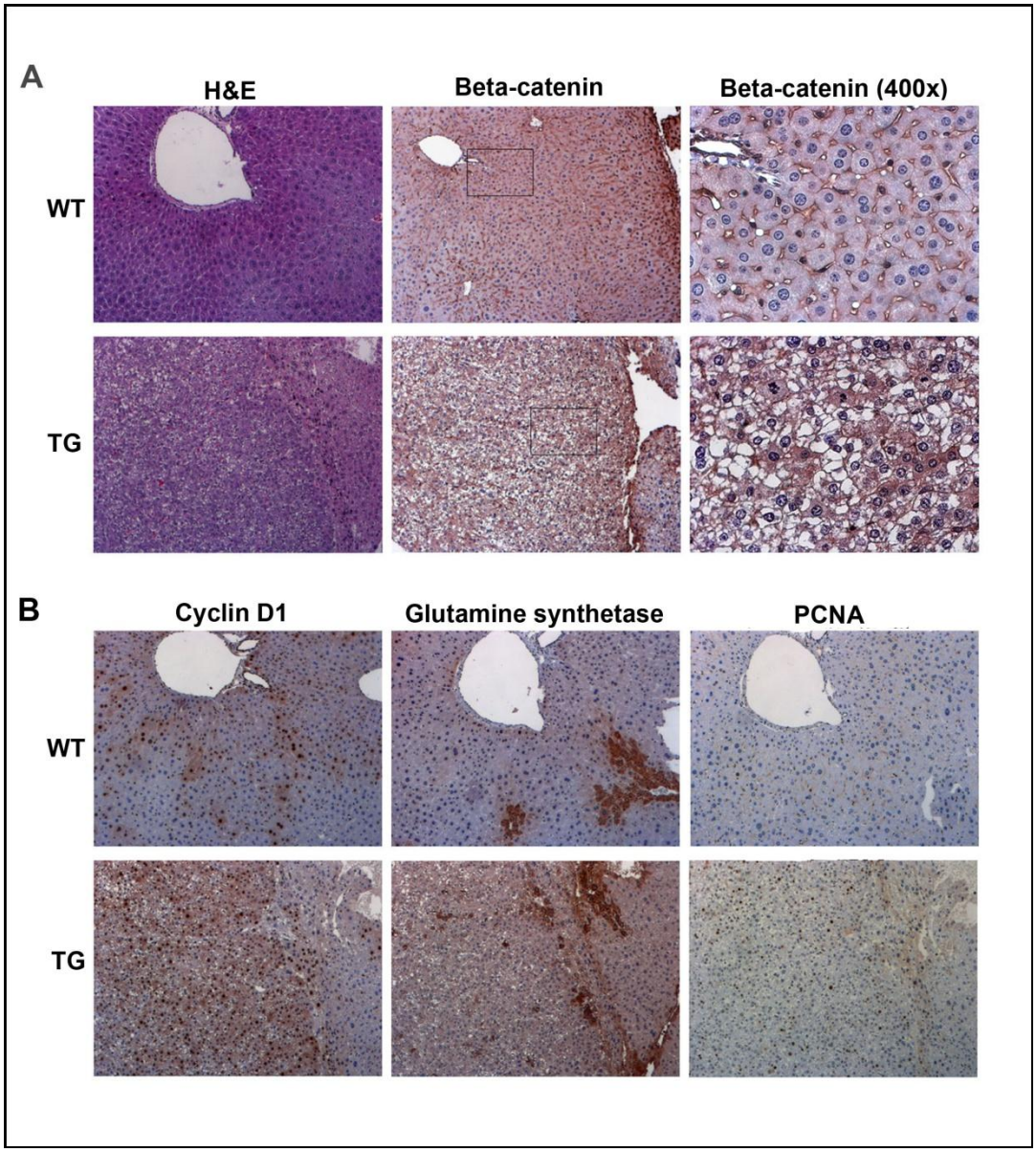


Figure 7: Activation of Wnt pathway in TG tumors at 6 months after exposure to DEN.

(A) H&E staining shows tumor formation in TG and not WT at 6 months after exposure to DEN. Consecutive sections show IHC for β-catenin localizing to cytoplasm and nuclei in TG tumors while membranous localization is evident in WT. **(B)** IHC for cyclin D1, GS and PCNA in consecutive sections from TG and WT liver at 6 months after DEN. Increased cyclin D1 is evident in TG tumor, while GS shows typical peri-central localization as compared to WT. Increase PCNA-positive cells are evident in TG tumors only. Images at 100x unless indicated.

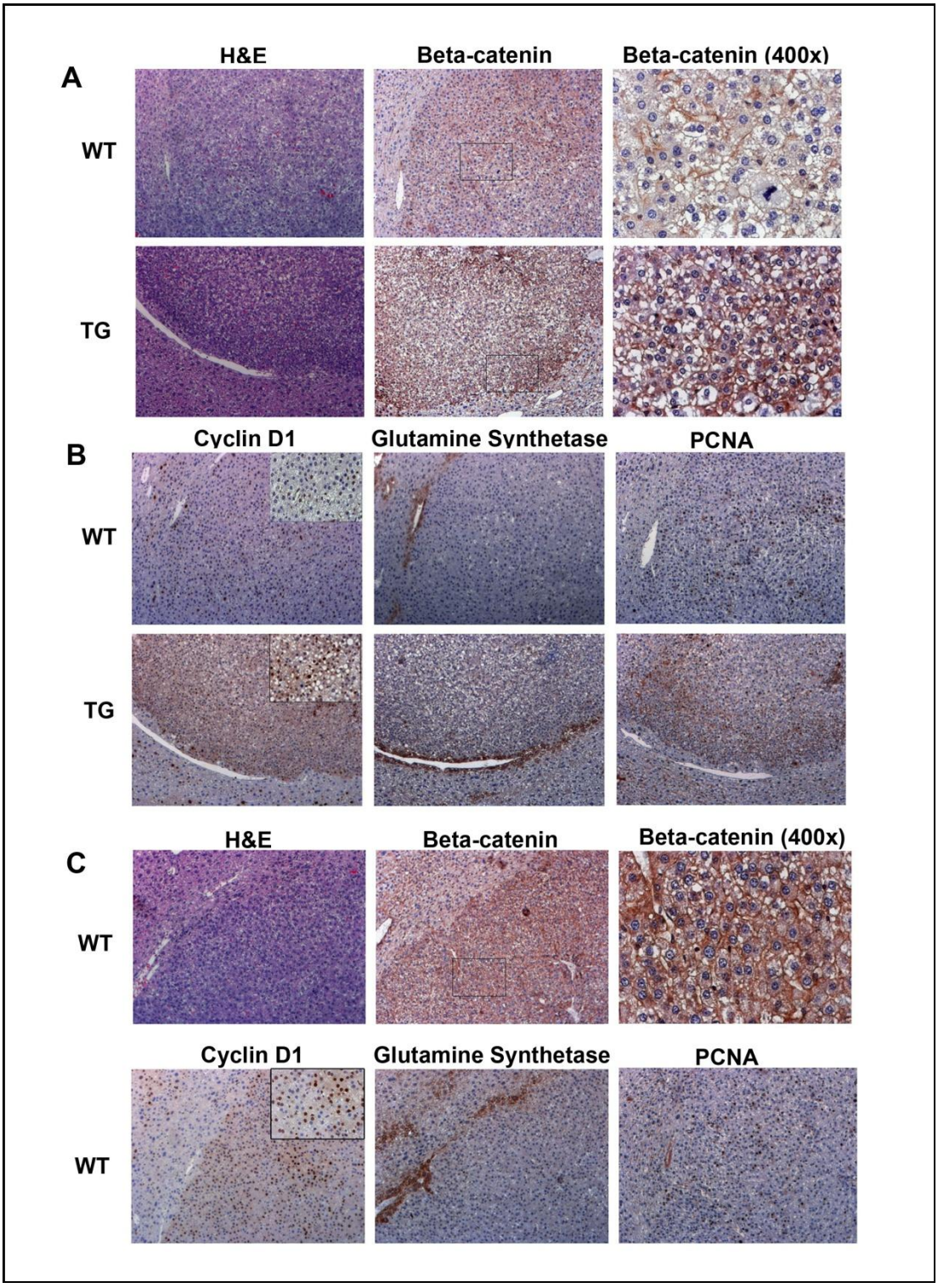


Figure 8: Activation of Wnt pathway in TG tumors at 9 months after exposure to DEN.

(A) H&E staining shows tumor formation in both WT and TG mouse liver 9 months after DEN exposure. Consecutive sections show IHC for β-catenin, which localizes predominantly to membrane in WT tumors and in

addition in cytoplasm and nuclei in TG tumors. Images at 100x magnification **(B)** IHC on consecutive sections show greater numbers of cyclin-D1 and PCNA-positive cells in TG tumors as compared to WT, while GS localization continued to be peri-central and unaltered in tumors. Images at 100x magnification. Inset for cyclin-D1 is at 400x magnification. **(C)** IHC for β-catenin and cyclin-D1 show activation of Wnt pathway in a minority of WT tumors as shown in a representative group. Consecutive sections show histology by H&E and nuclear/cytoplasmic β-catenin and increased numbers of cyclin-D1 and PCNA-positive cells by IHC. These tumors were negative for GS, which showed peri-central staining. Images at 100x magnification. Inset for cyclin-D1 is at 400x magnification.

3.3.5 Novel molecular players in DEN-induced TG liver

To examine what other pathways may be involved in early tumorigenesis in TG mice; we performed gene array analysis on liver samples from WT and TG mice at 6 months after DEN exposure (Table 5). We report several select genes that were differentially up or down regulated in the TG livers when compared to WT liver and might further our understanding of the mechanisms by which β-catenin might be inducing tumorigenesis. Additional studies will be essential to address their relative significance in tumorigenesis.

Table 5: List of select genes that are up or down regulated in TG versus WT at 6 months after DEN

Upregulated Genes	Fold Change	Downregulated Genes	Fold Change
Lipocalin 2	12.47	Carbonic Anhydrase 3	0.11
Calgranulin A	5.18	ATP5b	0.16
Cyp2b13	4.30	Apolipoprotein M	0.17
Igfbp5	3.47	Slc10a1	0.23
Calgranulin B	3.21	Slc25a11	0.24
FGF22	2.97	Syndecan1	0.24
Foxp1	2.92	IGFBP4	0.25
Sfrp1	2.90	FABP5	0.26
Calcyclin	2.76	FGF1	0.28
Metallothionen 1	2.65	Thioredoxin 2	0.28
Jun oncogene	2.60	Transferrin	0.32
Metallothionien 2	2.53	Cdc42	0.33
Gro1oncogene	2.53	Cyclin I	0.35
Peroxiredoxin 1	2.39	Hsp68	0.36
Cyp2b9	2.22	RhoA	0.38
Ribosomal protein L3	2.21	Jak1	0.42
Vimentin	2.21	Cathepsin H	0.43
Serum amyloid A2	2.19	Hsp60	0.43
PPAR gamma coactivator-1beta protein	2.17	EIF4e	0.44
S100 calcium binding protein A10	2.14	LKB1	0.51

3.4 DISCUSSION

β-Catenin is the crucial downstream effector of canonical Wnt pathway, and its activation is essential in liver development and regeneration (194). Mutations in *CTNNB1* that render its

protein stable, such as mutations affecting serine-45, have been implicated commonly in HCC (125). Both non-phosphorylatable and phospho-mimetic mutations affecting serine-45 along with others in exon-3 have been shown to retard degradation of β-catenin protein (8). It is relevant to note that none of the mutations affecting serine-45 alone (S45D, S45A or S45F) sufficiently enhanced β-catenin activation. We generated hepatocyte-specific TG mice over expressing S45D-β-catenin mutant and characterized them for hepatocyte proliferation, liver regeneration and hepatocarcinogenesis.

It was intriguing to note a lack of any overt phenotype or any detectable abnormalities in metabolism, zonation or persistent proliferation in TG mice over expressing S45D-β-catenin. The modest increase in LW/BW ratio at 1-month was transient and secondary to increased cyclin-D1 expression and proliferation. By 2 months of age, the TG liver adapted to and successfully monitored excessive β-catenin by sequestering it at the hepatocyte membrane in complex with E-cadherin and met. This unique means of turning "off" β-catenin signaling is under investigation and identification of molecules, which promote such sequestration, will have novel therapeutic implications in the treatment of β-catenin-active tumors in the liver and elsewhere. Indeed, membranous localization of dephosphorylated β-catenin in response to Wnt signaling has been suggested as an alternate output of Wnt signaling (197).

Despite successful sequestration of β-catenin at the hepatocyte membrane TG hepatocytes show a growth advantage both *in vitro* and *in vivo*. This indicates that when suitable signals are provided, the excess β-catenin from its membranous pool is available for transactivation of target genes. Indeed in primary cultures, the growth advantage of TG hepatocytes is due to increased availability of β-catenin from the membrane due to presence of

growth factors such as HGF and EGF in the culture media (198). Similar growth advantages of TG hepatocytes were also visible *in vivo* after PH.

β-Catenin is implicated in development of clinical and preclinical liver tumors due to mutations in *CTNNB1* or its regulators, which are evident in a subset of hepatic adenomas, HCC, hepatoblastomas, and hepatic tumors in DEN-exposed mice (199). Intriguingly, no study to date has shown spontaneous HCC in TG mice over expressing wild-type or constitutively active β-catenin (89, 90, 134). Interestingly the animal model that succumbs to spontaneous HCC is the conditional APC deletion; however APC deletions are not reported in HCC patients (131, 132). APC has since been shown to have direct DNA-binding functions through which it inhibits cell proliferation (200). Similarly, deletion of exon-3, while not reported in HCC, when used to generate transgenic mice, lacked spontaneous tumorigenesis, however, when combined with H-ras mutation, induced hepatic tumors (134, 135). Akin to these observations, spontaneous HCC was undetectable in our TG mice. In light of the observed membranous sequestration of β-catenin, the phenotype or lack thereof, in TG mice is not surprising. Nonetheless, as observed in primary cultures or after PH, DEN exposure also accelerated tumorigenesis in TG mice. Thus, it appears that mutation in *CTNNB1* alone is insufficient to cause spontaneous HCC and suggests the requirement of a "second hit", such as chemical induction in tumorigenesis.

The tumors in TG livers showed nuclear β-catenin, cyclin-D1 and increased proliferation, while GS was mostly unaltered. It was recently reported that hepatoblastomas harboring β-catenin mutations could be segregated into two subsets (100). A more mature subset of hepatoblastomas showed upregulation of target genes, which are commonly associated with zonality, i.e. GS. A second subset showed upregulation of progenitor-associated targets, such as c-myc and hepatoblast marker, α-fetoprotein. It will also be interesting to explore the possibility

of HCC harboring *CTNNB1* mutations to demonstrate heterogeneity in target gene expression based on extent of differentiation. It should be noted that heterogeneity in target gene expression based on mechanism of β-catenin activation is already evident in HCC (104, 201).

How β-catenin activation induces HCC remains elusive. The current model might allow elucidation of such mechanisms. We utilized gene array approach to compare genetic changes in livers of DEN-exposed 6M-old TG and WT mice. Several genes associated with HCC were upregulated, including insulin-like growth factor binding protein 5 (*IGFBP5)* and *JUN* oncogene (202, 203), and both have been shown to be regulated by β-catenin (204, 205). We also observed downregulation of various genes of relevance in tumorigenesis. *LKB1* expression was downregulated and its loss in exon-3-deleted β-catenin over expressing mice, accelerates HCC development (206). Cyclin I expression was decreased and is a negative cell cycle regulator (207). Additional studies will be necessary to validate these findings as a mechanism of β-catenin driven hepatocarcinogenesis.

3.5 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Findings

- Over expression of serine-45 mutated β-catenin provided a modest growth advantage post-natally as evidenced by increased liver weight and hepatocyte proliferation.
- The growth advantage becomes regulated by 2 months and the regulation seems to be via membranous sequestration of excess β-catenin with its binding partners E-cadherin and Met.

- Spontaneous hepatic tumorigenesis does not occur after over expression of serine-45 mutated β-catenin
- Hepatic tumorigenesis in response to a single injection of DEN is promoted by over expression of serine-45 mutated β-catenin as evidenced by earlier tumor development in TG.
- Tumor development appears to involve canonical Wnt signal as enhanced expression of the Wnt target cyclinD1 is observed in tumors.

Future Directions

- Explore mechanisms of membranous sequestration of point mutated β-catenin.
- Explore the role of targets identified in gene array as up or down-regulated in TG 6 months after DEN as potential therapeutic targets.
- Test therapies directed at the Wnt pathway in our model prior to gross tumor development to see if enhanced tumor development can be delayed or eliminated.
- Treat TG mice with Wnt targeted therapies starting at 6 months after DEN exposure to see if such therapy can stop the growth or even decrease the size of existing tumors.

4.0 DISPARATE CELLULAR BASIS OF ALLEVIATION OF INTRAHEPATIC CHOLESTASIS IN β-CATENIN OVEREXPRESSING MICE AFTER LONG-TERM DDC EXPOSURE

4.1 ABSTRACT

Administration of hepatotoxic diet containing 0.1% 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) induces biliary followed by hepatocyte injury, which is repaired through atypical ductular proliferation (ADP), oval cells and their subsequent differentiation to cholangiocytes and hepatocytes. Here, we examine whether presence of excess β-catenin in transgenic (TG) mice would provide any reparative advantage in response to DDC. No differences in appearance and numbers of total A6-positive oval cells were observed after DDC administration. However, an increase in A6-positive 'atypical hepatocytes' in the TG livers is observed at 14 and 28 days that coincided with an increase in PCNA-positive hepatocytes. Intriguingly, after chronic DDC administration for 150 days, a further increase in atypical hepatocytes is evident in TG, with higher numbers of PCNA positive hepatocytes exhibiting cytoplasmic/nuclear β-catenin and α-fetoprotein but not CK19, HNF1β, or Trop-2. Coincidently, we observe improved bile duct histology and a decrease in serum bilirubin and alkaline phosphatase levels in the TG mice indicating an enhanced resolution of intrahepatic cholestasis. TG mice exposed to DDC for 4 weeks followed by 2 days of normal chow showed decrease in

alkaline phosphatase, ADP, and periportal inflammation compared to WT, verifying improved recovery in the TG livers. Thus, we report a potential role of β-catenin over expressing hepatocytes in improved resolution of intrahepatic cholestasis after DDC-induced hepatic injury.

4.2 BACKGROUND

Despite being first identified more than 50 years ago, adult liver progenitor cells or oval cells remain an enigma. Little is known about the molecular mechanisms that drive their activation and proliferation. While such cells are scarce in healthy liver, they can be found adjacent to the terminal ducts of the biliary tree (147). These cells are generally quiescent and only arise in situations where the innate proliferative capacity of the hepatocytes and cholangiocytes is impaired or overwhelmed. Indeed, oval cell activation is observed in conditions associated with chronic liver injury and development of hepatocellular carcinoma (HCC) such as chronic viral hepatitis, alcoholic liver disease, and nonalcoholic fatty liver disease (150, 162). Greater than 50% of human HCCs express one or more markers of progenitor cells or oncofetal proteins such as α-fetoprotein, cytokeratins 7, 14 or 19 (163-165). This is even more relevant since around half of the small cell dysplastic foci, the earliest cancer precursor lesions known to date, consist of progenitor cells and their progeny, suggesting that these lesions are the result of activation and proliferation of progenitor cells (166). Another report has shown that half of hepatocellular adenomas consist of progenitor cells and intermediate hepatocytes (167). Given such association with liver cancer, great interest lies in identification of the molecular characteristics of this cell population given that these cells may be potential targets for therapy.

One pathway that likely plays a role in the oval cell response is the Wnt/β-catenin pathway. β-Catenin translocates to the nucleus and activates transcription of various target genes following binding of Wnt ligand to frizzled at the cell membrane and a series of phosphorylation events that lead to the dissociation of β-catenin from the Axin/Apc/GSK3β degradation complex. Previous studies have shown a conclusive role for β-catenin in hepatocyte maturation and differentiation during hepatic development (78, 80, 81). We have reported that immature hepatocytes or progenitors possess greater nuclear and cytoplasmic localization of β-catenin in comparison to more differentiated hepatocytes, where β-catenin is mostly located at the hepatocyte membrane in association with E-cadherin and c-Met, which was also evident during matrigel induced hepatocyte differentiation in primary cultures (82). Others and we demonstrated the direct importance of Wnt/β-catenin signaling in hepatic oval cell biology in rat and mouse models (Figure 5)(177, 178).

Various models are currently utilized to study the characteristics of oval cells in vivo, including the DDC diet model used in mice (159, 160). Chronic DDC-exposure leads to a significant hepatic injury, with ongoing repair prompted by atypical ductular proliferation and oval cells or hepatic progenitors (160, 171). In the current study we compare and contrast the response of control or β-catenin over expressing mice to short-term (4 weeks) and long-term (150 days) DDC-exposure. Interestingly, we found that β-catenin over expression in hepatocytes led to improved resolution of intrahepatic cholestasis that was coincident with an increase in A6-positive hepatocytes and greater number of patent bile ducts following long-term DDC exposure. This suggests an overall greater ability of the transgenic livers to resolve the DDC-induced hepatic injury especially by enhanced biliary repair.

4.3 RESULTS

4.3.1 Cellular disparity among the A6-positive population despite comparable ADP in WT and TG after short-term DDC exposure

Transgenic (TG) and wild-type (WT) mice in FVB background were placed on DDC diet for time periods ranging from 3-28 days to examine the ADP response in the presence of excess β-catenin. No differences in histology and serum biochemistry measurements for bilirubin, AST, and ALT were observed between the two groups (Fig.9a and data not shown). Initial analysis of the response to DDC was performed by staining for A6, a ductular and progenitor marker. Surprisingly, we observed no change in the number of A6 positive cells between WT and TG mice between 3 and 28 days (Fig. 9a,b). We did, however note a disparity in the morphology of A6 positive cells between the two groups at 14 and 28 days (Fig. 9a). While a majority of the A6 positive cells in WT took on a typical ductular appearance, we found that some of the A6 positive cells took on the morphology of a hepatocyte. Furthermore, the number of A6 positive hepatocytes (denoted "atypical hepatocytes" in the rest of this study) appeared to be greater in the TG liver. Indeed, quantification of only atypical hepatocytes showed a significant increase at 14 days and, more notably, 28 days in the TG liver (Fig. 9c). Coincidentally, there is an increase in PCNA positive hepatocytes, but not biliary epithelial cells, in the TG compared to the WT at 28 days suggesting that the atypical hepatocytes may exhibit a greater proliferative capacity (Fig 10a-c). No differences in fibrosis were observed between the two groups over 3-28 days of DDC diet feeding as measured by trichrome staining (Fig. 10a).

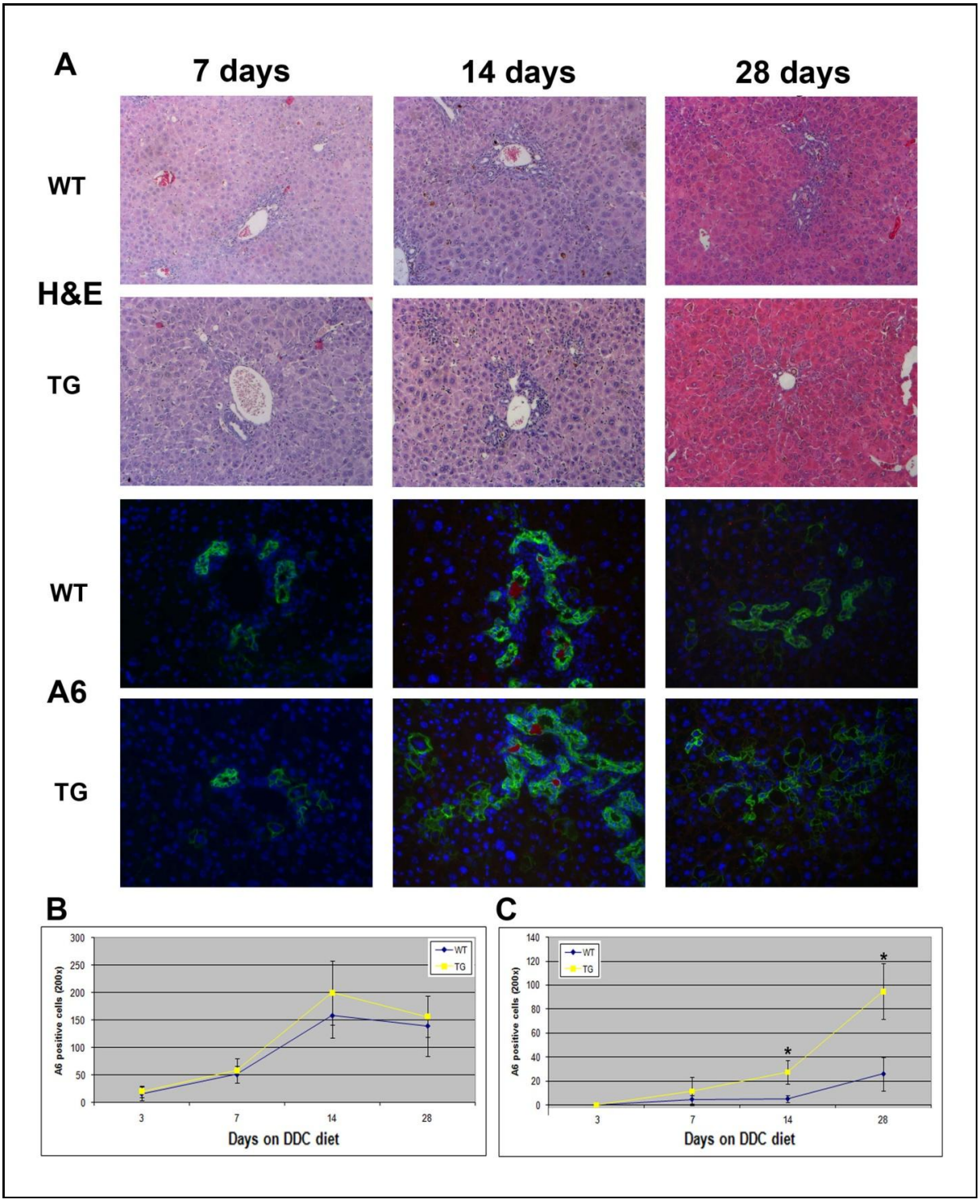
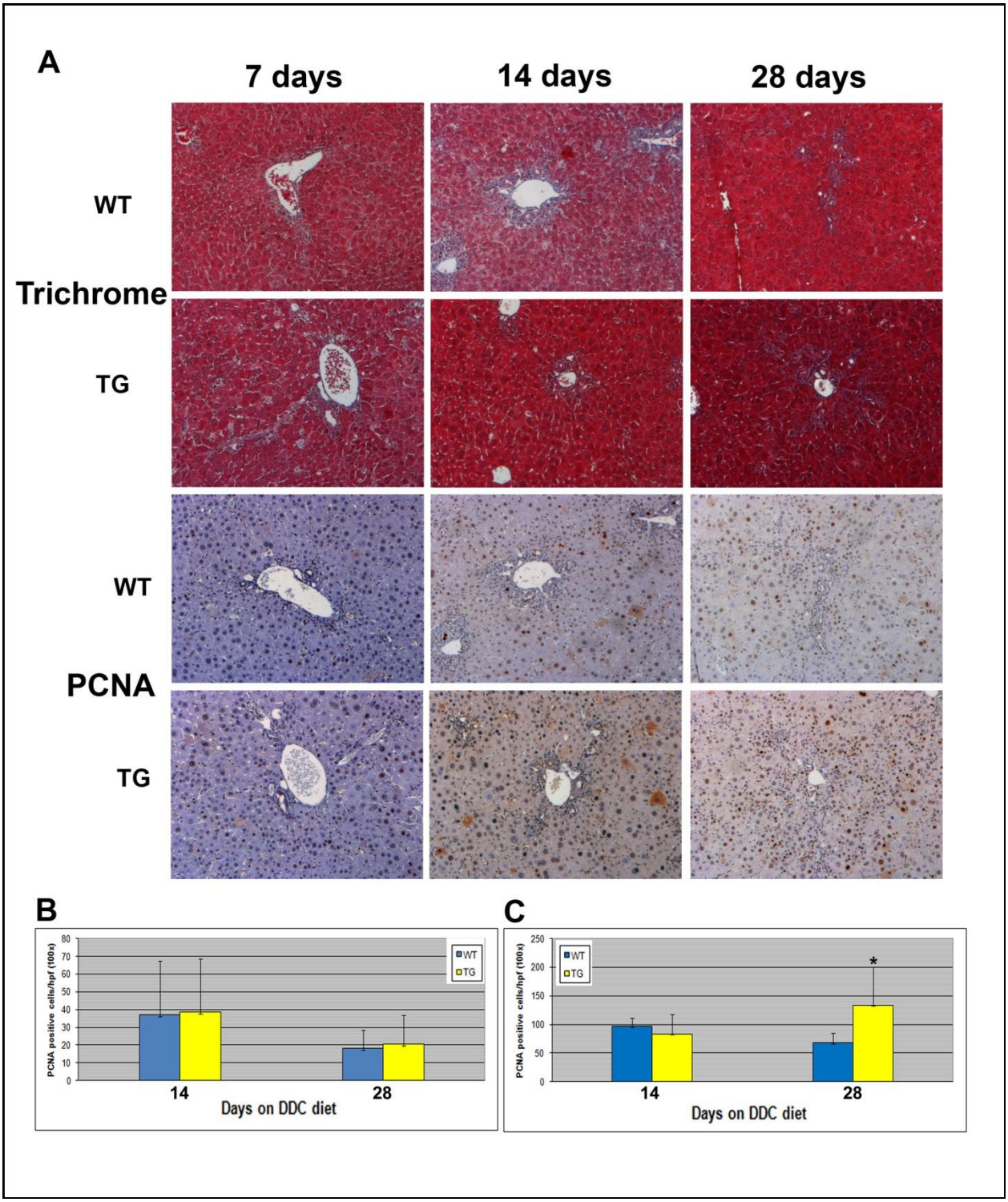


Figure 9: No change occurs in overall ductular response to DDC with over expression of β-catenin, but presence of morphological disparity among A6 positive population, especially in number of A6-positive or atypical hepatocytes.

(A) Representative photomicrographs of H&E and A6 immunofluorescence staining (green) of liver from WT and TG after 7, 14, and 28 days of feeding with DDC diet. H&E at 100x magnification, A6 at 200x magnification. DAPI (blue) was used to stain nuclei in immunofluorescence images. **(B)** Quantification of total A6 positive cells in WT and TG after 3, 7, 14, and 28 days of DDC feeding. No significant difference was observed at any of the time points between WT and TG. **(C)** Quantification of A6 positive hepatocytes in WT and TG after 3, 7, 14, and 28 days of DDC feeding. More A6 positive hepatocytes were observed in TG compared to WT at 14 and 28 days. *-$p<.01$

Figure 10: Increase in hepatocyte proliferation, but no change in fibrosis observed in TG after short term DDC exposure.

(A) Representative photomicrographs for trichrome (fibrosis, blue) and PCNA staining (proliferation, brown) of liver from WT and TG after 7, 14, and 28 days of feeding with DDC diet at 100x magnification. **(B)** Quantification of PCNA positive biliary epithelial cells at 14 and 28 days of DDC feeding shows no difference between WT and TG. (C) Quantification of PCNA positive hepatocytes at 14 and 28 days of DDC feeding shows a significant increase in TG at 28 days compared to WT. *-$p<.01$


A
7 days
14 days
28 days
WT
Trichrome
TG
WT
PCNA
TG
B
PCNA positive cells/hpf (100x)
WT
TG
14
28
Days on DDC diet
C
PCNA positive cells/hpf (100x)
WT
TG
*
14
28
Days on DDC diet

4.3.2 Long-term exposure to DDC leads to high number of atypical hepatocytes in TG

We next placed mice on the diet for an extended period of time (150 days) to examine if the appearance of atypical hepatocytes would continue and whether or not this response would have any effect on the chronic injury or repair process. After long-term exposure to DDC in WT, we observed significant periportal and pericellular fibrosis along with abnormal levels of bilirubin and alkaline phosphatase indicating continued biliary injury (Fig. 11a, Table 6). Intriguingly, while WT livers do show the presence of atypical hepatocytes after 150 days of feeding DDC diet, a striking increase in their numbers was apparent in the TG livers (Fig. 11a). Upon quantification, we observe nearly twice as many atypical hepatocytes in TG liver compared to WT (Fig. 11b). In addition, while such hepatocytes were restricted to periportal areas in WT livers, their location was more widespread in the TG. Further analysis revealed several of such atypical hepatocytes to be exhibiting cytoplasmic and nuclear localization of β-catenin in the TG livers, a finding not observed in WT (Fig. 11a).

Table 6: Serum biochemistry after 150 days of DDC diet feeding (*p<0.05)

Serum Biochemistry		
Total Bilirubin	***WT***	***TG***
1	0.5	0.7
2	2.9	0.2
3	0.4	0.1
4	5.1	0.8
5	3.7	0.5
Average	**2.52**	**0.46**

Alkaline Phosphatase	***WT***	***TG***
1		1397
2	2323	1143
3	2001	1113
4	2199	1748
5	3093	1935
Average	**2404**	**1467.2***
AST	***WT***	***TG***
1	2223	2749
2	2134	1332
3	1877	1703
4	2256	2649
5	2597	2590
Average	**2217.4**	**2204.6**
ALT	***WT***	***TG***
1	2850	3673
2	2544	1219
3	2068	1966
4	2675	2914
5	3223	2597
Average	**2672**	**2473.8**

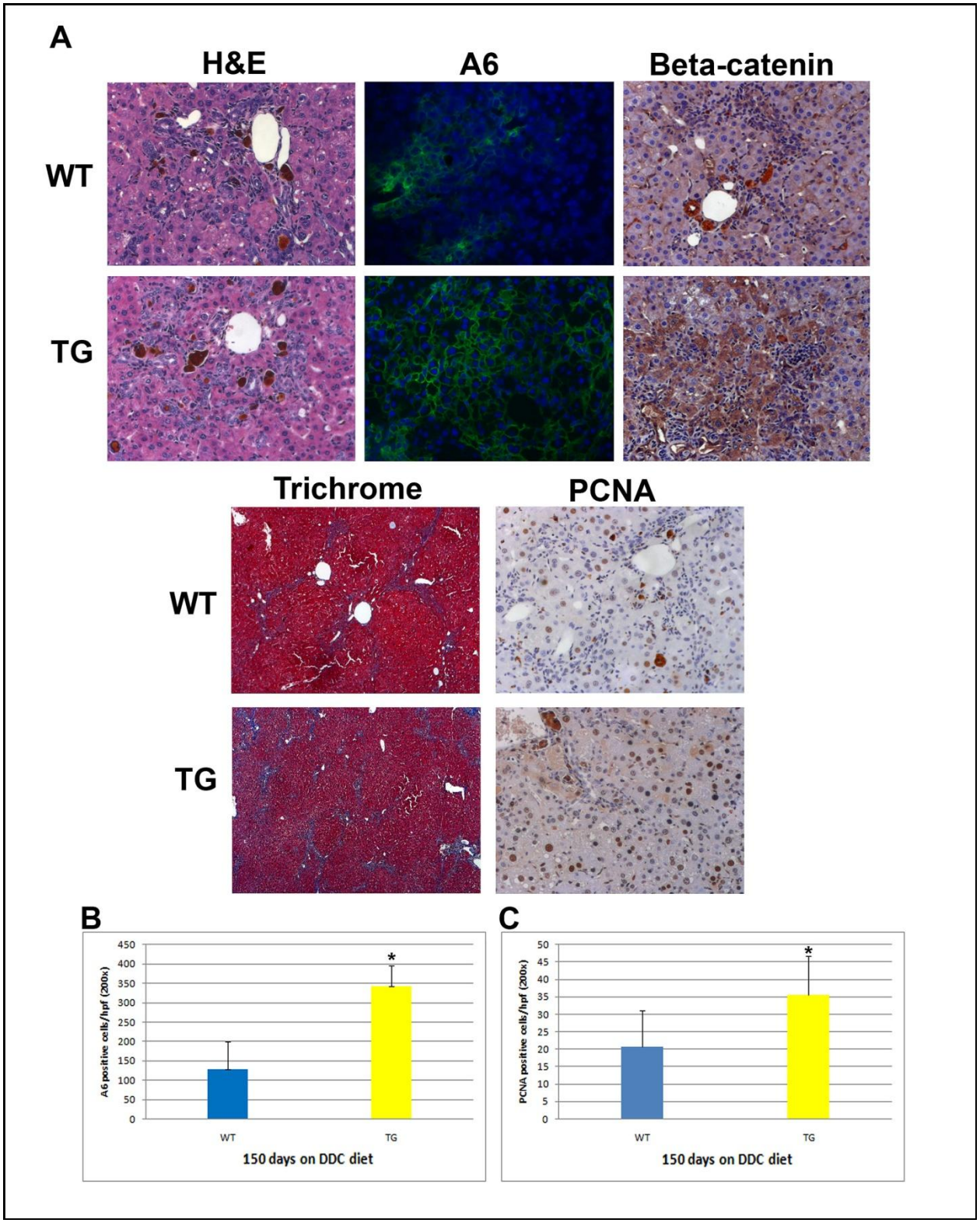


Figure 11: Continued increase in atypical hepatocytes and a decrease in markers of biliary injury in TG after long-term DDC feeding.

(A) Representative photomicrographs for H&E, A6 staining, trichrome, and PCNA staining of liver from WT and TG after 150 days of DDC diet feeding. H&E, A6 and PCNA at 200x magnification. Trichrome at 50x magnification. DAPI (blue) was used to stain nuclei in immunofluorescence images. **(B)** Quantification of A6 positive hepatocytes at 150 days of DDC feeding shows a significant increase in TG compared to WT. **(C)** Quantification of PCNA positive hepatocytes at 150 days of DDC feeding shows a significant increase in TG compared to WT. *-p<.01

4.3.3 Increased atypical hepatocytes in TG liver is associated with decrease in intrahepatic cholestasis after long term DDC feeding

To address any associated phenotypic association of enhanced atypical hepatocyte response to the chronic injury in the TG livers, we performed further histological analysis and serum biochemistry. Coincident with atypical hepatocytes, we observed greater hepatocyte proliferation in the TG livers as measured by PCNA staining suggesting that these cells have an increased capacity for proliferation and regeneration (Fig. 11a, c). Interestingly, serum analysis revealed that while bilirubin was abnormally elevated in 3 out of 5 WT animals, all of the TG animals (n=5) exhibited completely normal levels of total bilirubin (Table 6). Likewise, serum alkaline phosphatase levels are decreased by approximately 40% in the TG mice when compared to WT at 150 days (Table 6). In contrast to an improvement in markers of biliary injury, no change in serum measurements of hepatocyte injury, AST and ALT was observed (Table 6). We also examined the WT and TG livers at this stage for hepatic fibrosis through Masson Trichrome staining, which exhibited unremarkable differences between the two groups (Fig. 11A). Taken together, the above findings suggest that over expression of β-catenin in hepatocytes, which leads to an enhanced atypical hepatocyte response to the long-term exposure to DDC, played a specific role in aiding the repair of biliary injury.

4.3.4 TG livers resolve DDC-induced injury more rapidly after reinstitution of normal chow

To further verify the effect of β-catenin transgene on hepatic injury and resolution, we performed DDC diet recovery studies. It was previously reported that the prominent biliary injury observed as ductular reaction, periductal inflammation, and abnormal serum biochemistry indicating intrahepatic cholestasis with DDC feeding resolves after the animal is placed back on normal chow (176). We placed TG and WT mice on DDC diet for 28 days at which time it was substituted with normal chow for 2 days. Such a switch led to normalization of serum bilirubin levels in both the WT and TG, although the values were consistently lower in the TG (Table 7). Additionally, serum alkaline phosphatase levels were significantly lower in TG compared to the WT mice (Table 7). To determine if the decline in biliary dysfunction also led to histological improvement we compared extent of ductular proliferation and periportal inflammation in WT and TG livers after cessation of DDC diet for 48 hours. Analogous to serum biochemistry, there was a decrease in ADP in the TG as compared to WT livers by H&E (Fig. 12A). This was verified by immunofluorescence that showed a dramatic decrease in A6 positive atypical ductular cells in the TG livers (Fig. 12a, c). We also observed ongoing periportal inflammation in the WT liver, whereas periportal inflammation had subsided in TG. This was confirmed by CD45 staining which showed many less infiltrating cells in the periportal zone of the TG livers (Fig. 12a, d). Interestingly, TG and WT livers continued to show ongoing hepatocyte injury with serum AST and ALT levels being around 15-18 fold higher than normal (Table 7). These findings reiterate that over expression of β-catenin in the hepatocytes provided a greater capacity to resolve intrahepatic cholestasis likely through repair and regeneration of the bile ducts.

Table 7: Serum biochemistry for DDC-induced injury recovery study ($p<0.05$)

Serum Biochemistry		
Total Bilirubin	***WT***	***TG***
1	0.6	0.2
2	0.4	0.2
3	0.2	0.2
4	0.3	0.1
Average	**0.375**	**0.175**
Alkaline Phosphatase	***WT***	***TG***
1	1269	928
2	1122	902
3	1108	996
4	986	809
Average	**1121.25**	**908.75***
AST	***WT***	***TG***
1	737	596
2	675	531
3	875	697
4	621	633
Average	**727**	**614.25**
ALT	***WT***	***TG***
1	778	681
2	622	666
3	993	738
4	726	831
Average	**779.75**	**729**

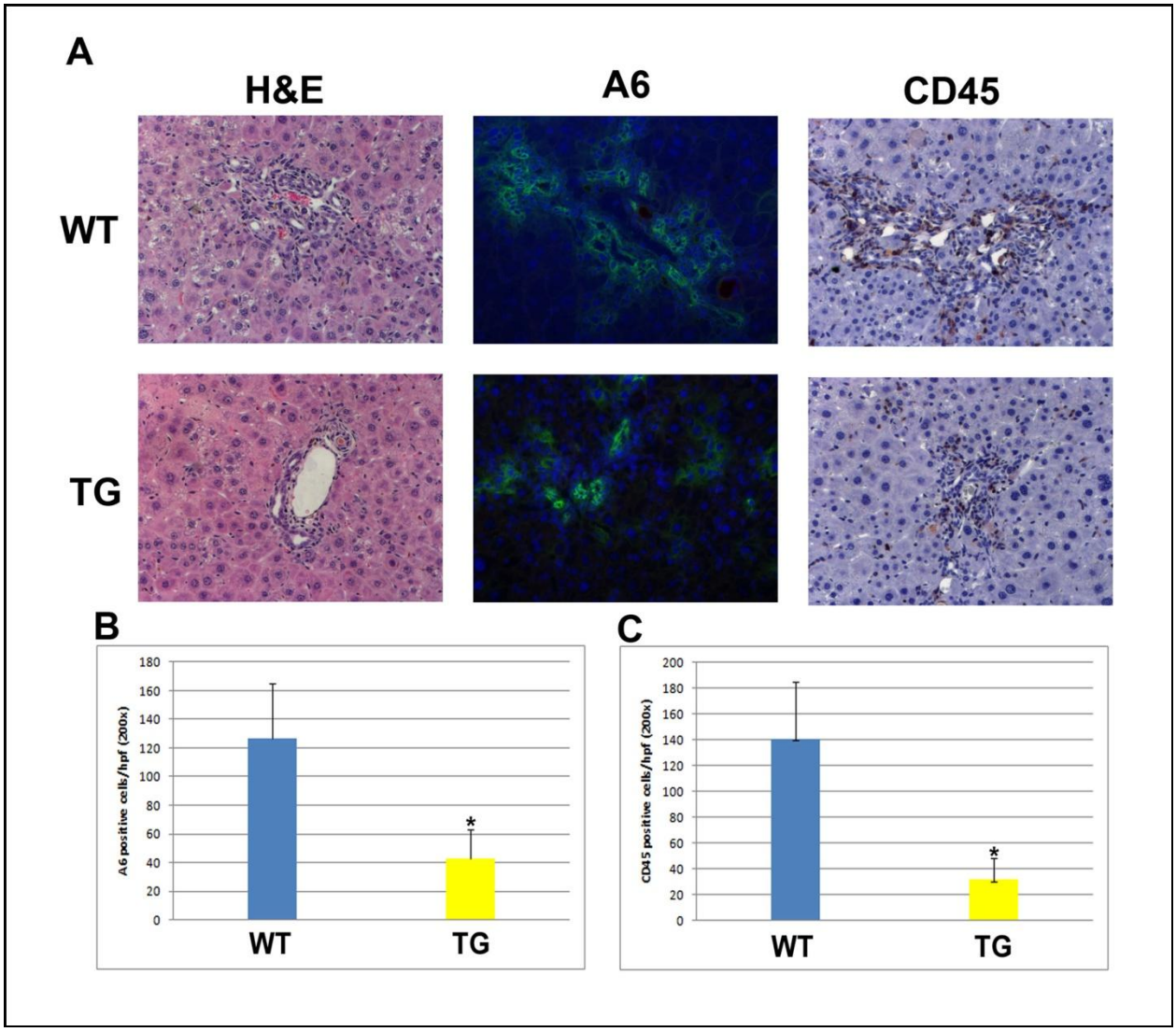


Figure 12: Over expression of β-catenin enhances recovery from DDC diet induced biliary injury. DDC diet was fed to mice for 28 days & substituted with normal chow for 48 hours.

(A) Representative photomicrographs for H&E, A6 staining, and CD45 staining of liver from WT and TG after 48 hours of recover on normal diet. All images at 200x magnification. **(B)** Quantification of A6 positive ductular cells shows a decrease in TG compared to WT. **(C)** Quantification of CD45 positive cells shows a decrease in TG compared to WT. *-p<.01

4.3.5 Analysis of differentiation markers after long-term DDC

To further address the role of cellular disparity in the form of increased numbers of atypical hepatocytes in the TG in promoting resolution of biliary injury, we began to examine various markers of differentiation in the TG and WT liver. Hepatocytes do not normally express the marker A6, which in the adult liver is only expressed in the biliary epithelial cells. However, the abundance of A6-positive hepatocytes in TG led us to believe that the differentiation status of these cells is altered. First, we examined expression of α-fetoprotein (AFP), a marker found in fetal hepatocytes, after long-term DDC exposure. Both WT and TG show expression of AFP in hepatocytes, but expression is clearly greater in TG (Fig. 13a). Interestingly, proliferating ductular cells do not express AFP. In contrast to AFP, Trop-2, a marker specific to the oval cell, was found predominantly in the reactive ductular epithelium in TG with only a few periportal hepatocytes showing expression of Trop-2 (Fig. 13a) (208). Alternatively, Trop-2 is present in both proliferating ductular epithelium and periportal hepatocytes of WT liver. Another marker that, much like A6, is commonly observed in both proliferating and quiescent ductular epithelial cells is CK19, which was examined here to see if it is also expressed in atypical hepatocytes. Intriguingly, CK19 was only expressed in ductular epithelial cells and not in hepatocytes (Fig. 13b). Furthermore, we noted that several portal triads in the WT did not have any visible CK19 positive bile ducts indicating that they succumb to continual injury (Fig. 13c). This was confirmed on H&E showing no lumen surrounded by biliary epithelial cells in such portal triads (Fig. 13c). However, TG liver showed continued presence of viable bile ducts (Fig. 13b). We also examined expression of HNF1β, a transcription factor observed in both the bipotential hepatoblast as well as mature biliary epithelial cells. Expression of this maker was predominantly observed in the ductular epithelium in both groups (Fig. 13b). WT hepatocytes

showed modest cytoplasmic staining for HNF1β while this marker was absent in periportal hepatocytes of TG. Thus, we observe diversity of marker expression in periportal hepatocytes between WT and TG indicating a difference in differentiation status and/or source of hepatocytes around the portal triad.

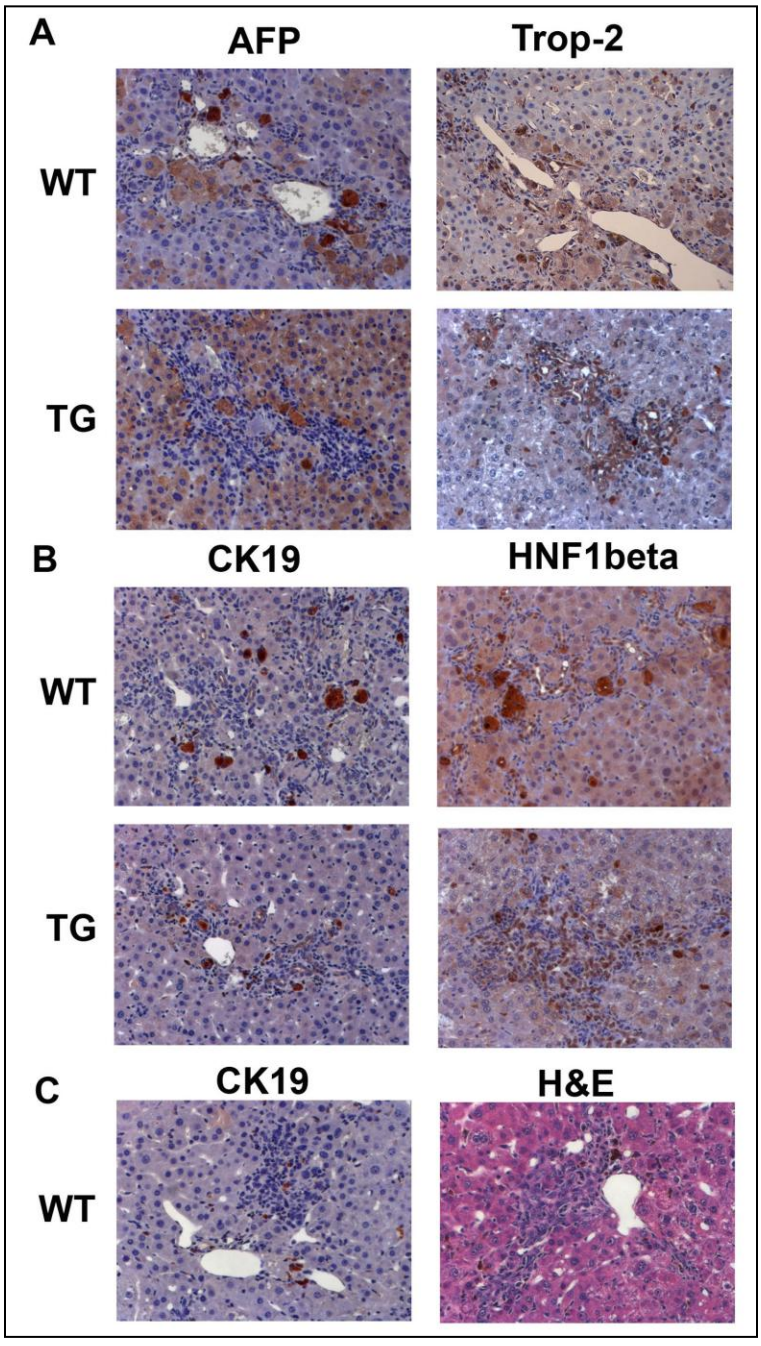


Figure 13: Expression analysis of markers of differentiation in liver after long-term DDC (150 days).

(A). Representative photomicrographs for α-fetoprotein (AFP) and Trop-2 staining of liver from WT and TG after 150 days of DDC feeding. All images at 200x magnification. **(B)** Representative photomicrographs for cytokeratin 19 (CK19) and hepatocytes nuclear factor 1β (HNF1β) staining of liver from WT and TG after 150 days of DDC feeding. All images at 200x magnification. **(C)** CK19 and H&E photomicrographs from WT liver representing the occasional finding that portal triads are devoid of mature bile ducts. Both CK19 and H&E are at 200x magnification.

4.4 DISCUSSION

In the current study we examined the response to short- and long-term DDC diet in our mouse model over expressing mutated β-catenin. The DDC diet model has been utilized in mice for various purposes including Mallory body formation, oval cell induction, and more recently was reported as model for inducing primary sclerosing cholangitis (159, 160, 171, 176, 209, 210). Following exposure to DDC, primarily biliary injury occurs followed by hepatocyte injury. The injury is associated with over expression of proinflammatory and profibrotic cytokines, elevation of markers of hepatic injury, periductal neutrophil infiltration, myofibroblast activation and proliferation, and subsequent periportal fibrosis. A diverse set of reparative responses in the form of appearance and differentiation of the hepatic progenitors or oval cells in addition to ductular proliferation, ADP and hepatocyte proliferation is evident in this model (160, 171). Thus, DDC-induced injury incites ADP along with an oval cell response and these oval cells continually differentiate into biliary epithelial cells and hepatocytes in an attempt to resolve and repair the hepatic damage. This allows for DDC diet to be compatible with life for at least 5-6 months, which were the longest time points examined in our study. Various specific or non-

specific markers for oval cells have been described, such as A6 and Trop-2, while others such as α-fetoprotein and albumin are less well characterized (159, 160, 171, 208, 211).

Despite the attempts at repair, and animals displaying near normal physical activity, chronic DDC exposure led to dramatically larger livers sometimes constituting up to 20% of body weight as has also been described elsewhere (160). Additionally, there is evidence of continuing abnormal serum biochemistry depicting chronic hepatic insult. In fact DDC exposure for 150 days displayed around 50-fold higher levels of serum AST and ALT levels in regular FVB mice. At the same time these animals show significant biliary injury as seen by histology and increased cholestasis reflected by around 24-fold higher levels of alkaline phosphatase and around 2.5-fold excess levels of total bilirubin.

In the current study we sought to explore how β-catenin over expression under the regulation of albumin promoter/enhancer in the hepatocytes might influence the oval cell response and how it might impact the overall repair process after chronic exposure to DDC through examination of histology and serum biochemistry. The premise behind the study was a previously reported role of β-catenin in oval cells in rat and mouse models by others and us (177, 178). In mice, β-catenin was shown to colocalize with the A6 population of cells and there was induction of multiple Wnt ligands after DDC-exposure. More definitively, blunted ADP and oval cell response was reported in the conditional β-catenin null liver. These findings led us to hypothesize that over expression of β-catenin may induce robust ADP which may lead to increased oval cell response after short-term exposure to DDC. Surprisingly, quantitative differences between the numbers of total A6 positive cells between the TG and WT were unremarkable from 3 to 28 days of DDC feeding. A possible explanation could be that β-catenin transgene is under the control of an albumin promoter-enhancer and oval cells may not express

albumin especially at the outset, which by itself is debatable (212, 213). However, while there are no dramatic differences in extent of ADP or oval cell numbers, we noted a clear morphological disparity between the A6 positive populations. The TG livers clearly exhibited a greater propensity towards A6-positive 'atypical hepatocytes'. The periportal presence of A6-positive hepatocytes in the immediate proximity of oval cells has been shown previously after DDC injury, and seems to represent the newly differentiated hepatocyte population from the oval cells, which are temporally retaining the A6 marker (160). Thus at 28 days of DDC treatment the numbers of atypical hepatocytes in TG were already significantly greater than WT emphasizing the evidence of cellular disparity in response to injury or recovery thereof, *albeit* at this stage no differences in serum biochemistry were apparent.

When DDC feeding was continued for a total of 150 days, most of the resident hepatocytes in the TG liver had become A6 positive. While WT livers also contained atypical hepatocytes, these were restricted to periportal areas and were far fewer in number than in TG where their location was more widespread within the liver. Intriguingly, at this time we observed greater improvement in the two markers of intrahepatic cholestasis, serum bilirubin and alkaline phosphatase, in the TG while markers of hepatocyte injury and fibrosis seemed to be unaffected by the presence of transgene. An additional piece of evidence in support of this is the finding that after chronic DDC some portal triads in the WT show a paucity of mature bile ducts with normal lumen, while TG liver more consistently contained mature and patent bile ducts in the portal triad. While the absence of bile ducts does not always occur in the WT, this disparity indicates that β-catenin over expression in TG livers may be promoting biliary repair through retention and maturation of bile ducts leading to resolution of cholestasis.

The above observations suggest that following DDC injury in TG liver, an increase in the numbers of atypical hepatocytes, which express A6, a marker common to oval cells and biliary epithelial cells, was evident (211). These cells expressed nuclear and cytoplasmic β-catenin and fetal hepatocyte marker α-fetoprotein, but lacked expression of specific oval cell marker Trop-2 and typical biliary markers- CK-19 and HNF1β. Thus, these hepatocytes are indeed atypical and appear to represent an intriguing cell type whose origin remains elusive, but they appear to be associated with greater resolution of intrahepatic cholestasis and improved bile duct phenotype in response to DDC. This phenomenon was also verified in the recovery studies where TG livers exhibited a greater capacity for repairing biliary epithelium after 4 weeks of DDC injury, at which time the atypical hepatocyte population was already significantly greater in TG. The injury resolution was reflected by significant decrease in inflammation, ADP and serum alkaline phosphatase levels in TG within 2 days of substitution of DDC diet by normal chow.

What might be the origin of A6-positive atypical hepatocytes? While lineage-tracing studies will be essential to definitively address this question, we are able to discuss the possibilities based on the current observations summarized above. In WT liver, in response to chronic DDC injury, the sequence of events appears to be the following (Figure 14): DDC injury induces ADP which led to the appearance of oval cells that become intermediate hepatocytes and finally differentiate into mature hepatocytes. The appearance of atypical hepatocytes was minimal here and appears to be most likely a result of temporal retention of some biliary markers such as HNF1β, Trop-2, and/or A6, and temporary acquisition of AFP. However, the appearance of increased A6 positive hepatocytes that are negative for HNF1β and Trop-2 but positive for AFP in TG suggests a rather unique phenotype. This may represent an attempt of the β-catenin over expressing transgenic hepatocytes to repair the primary biliary injury through

transdifferentiation into bile duct cells. Indeed, it has been reported that hepatocytes are capable of transitioning into biliary epithelial cells without dedifferentiating through a bipotential oval cell or hepatic progenitor (214, 215). Similarly, culture of embryonic livers in serum-free conditions in the presence of Wnt3a-conditioned media, resulted in survival of biliary epithelial cells only, but more importantly resulted in transdifferentiation of remnant hepatocytes into biliary epithelium without undergoing complete de-differentiation as well (86). It should also be mentioned that while hepatocyte proliferation is ongoing in both WT and TG hepatocytes during

	BEC		ADC		OC		AH		Hepatocytes	
	WT	TG	WT	TG	WT	TG	WT	TG	WT	TG
A6	+	+	+	+	+	+	+	++	--	--
CK19	+	+	+	+	+	+	--	--	--	--
Trop2	--	--	+	+	+	+	+	--	--	--
HNF1β	+	+	+	+	+	+	+	--	--	--
αFP	--	--	--	--	--	--	+	++	--	--

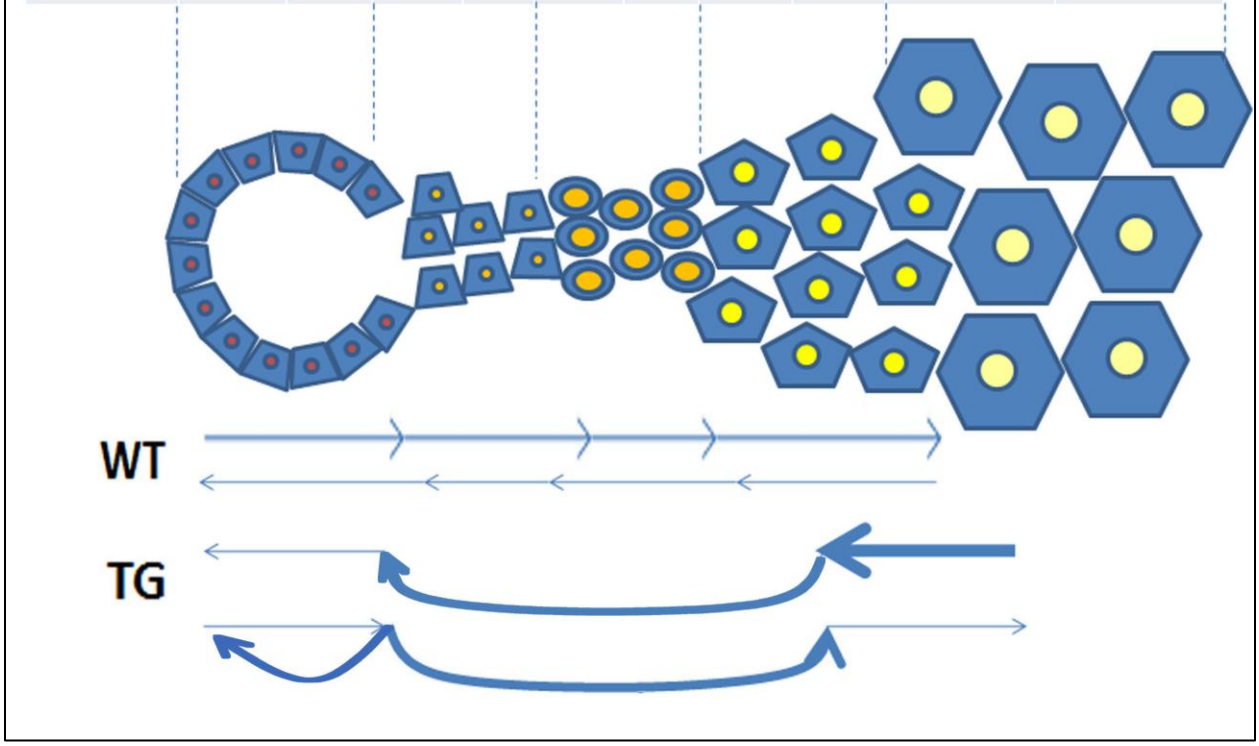


Figure 14: Cartoon depicting disparate mode of repair after long-term DDC-exposure in WT and TG livers. While bidirectional repair from surviving bile ducts and hepatocytes ensures survival after chronic DDC-injury that induces biliary and hepatocyte damage, the predominant mechanism appears to be through ductular proliferation, oval cell activation and transdifferentiation to hepatocytes in WT livers. In TG livers, however, increased numbers of A6, and AFP positive atypical hepatocytes, pan-zonally and lack of periportal retention of biliary markers indicates enhanced transdifferentiation of β-catenin-transgene expressing hepatocytes to biliary phenotype eventually resolving intrahepatic cholestasis. An alternate scenario of increased differentiation to biliary cells and 'spill over' to form A6+ hepatocytes upon expression of β-catenin transgene in the oval cells may also contribute to resolution of biliary injury. Abbreviations: BEC-biliary epithelial cell, ADC-atypical ductular cell, OC-oval cell, AH-atypical hepatocyte, CK19-cytokeratin 19, HNF1β-Hepatocyte nuclear factor 1β, and αFP-α-fetoprotein.

DDC administration as has also been reported elsewhere, the extent of proliferation was higher in the TG livers from the outset which is also coincident with increased A6/AFP positive hepatocytes (160). However, we cannot rule out that increased atypical hepatocytes may represent a 'spillover' of the spare de-novo-differentiated biliary cells that retain A6 similar to the situation in WT albeit more robust due to the presence of β-catenin transgene.

Indeed, previous studies in hepatic development have shown an important role of β-catenin in regulating differentiation of hepatoblasts towards a biliary lineage (78, 87). , These studies fit well with our observations that atypical hepatocytes in the TG livers fail to express Trop-2, which is a recently identified oval cell-specific marker (208). It is also conceivable that both of the above scenarios might be functioning in cohort to accelerate biliary repair in the TG mice and hence the overall phenotype might be an outcome of enhanced oval cell differentiation along with hepatocyte transdifferentiation to biliary epithelial cells.

The expression of biliary markers in hepatocytes has also been reported in various human diseases of the biliary tract. For instance, periseptal hepatocytes in cases of extrahepatic biliary atresia and alpha-1-antitrypsin deficiency show expression of the maker OV-6, a finding also

observed in hepatocytes from adult patients with primary biliary cirrhosis and sclerosing cholangitis (216, 217). It was also reported that hepatocytes in the setting of cholestasis begin to show expression of the bile duct marker cytokeratin 7 (CK7) (218). Interestingly, long-standing cholestasis led to an increase in the number of hepatocytes, which were expressing CK7, much like what we observe with A6 expression in hepatocytes after long-term DDC. This suggests that the appearance of hepatocytes expressing biliary makers increases with the amount of time the liver experiences biliary injury. Thus, chronic injury presents the need for more and more hepatocytes to be called in to action in the repair and regenerative process.

If increased activity of the Wnt pathway provides the liver with an advantage for repairing the biliary epithelium, be it through increased hepatocyte plasticity or some other mechanism, this may be of clinical significance in the treatment of diseases that involve chronic cholestatic injury as a primary or secondary component. It is possible that therapy to induce Wnt pathway activation in these patients could have some benefit for repair of the biliary injury caused by the cholestatic process. Future studies will be important to definitively characterize the role for β-catenin in biliary injury and repair. If this approach is eventually deemed feasible, studies must also weigh the benefits of inducing the Wnt pathway for injury repair versus the cost of potentially promoting tumor development given β-catenin's well-studied oncogenic role in liver cancer (199).

4.5 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Findings

- More atypical hepatocytes (positive for A6 and AFP, but negative for CK19, HNF1β, and Trop-2) appear in the TG from 14 to 150 days of DDC feeding than WT, along with an increase in periportal hepatocyte proliferation at 28 and 150 days.
- Lower serum bilirubin and alkaline phosphatase observed in TG compared to WT after 150 days of DDC feeding indicates improved resolution of intrahepatic cholestasis.
- More rapid recovery of normal histology and serum biochemistry in TG after DDC diet was replaced with normal chow.

Future Directions

- Perform lineage tracing studies to identify the source of atypical hepatocytes and to determine if their fate is to become biliary epithelial cells and/or hepatocytes.
- Perform hydrodynamic tail vain injection of Wnt-1 expression vector after DDC feeding to further verify that activation of the Wnt pathway induces better repair of the injury induced by the DDC diet.
- Subject TG and WT mice to another model of biliary injury, bile duct ligation, to see if the positive effects observed in the DDC injury model are universal to other biliary injury models or specific to DDC.
- Further examine the appearance of hepatocytes expressing biliary markers in samples taken from patients with biliary disease and study the status of the Wnt pathway in these human atypical hepatocytes.

5.0 SPONTANEOUS REPOPULATION OF BETA-CATENIN NULL LIVER WITH BETA-CATENIN POSITIVE PROGENITOR CELLS AND TUMOR FORMATION AFTER LONG-TERM EXPOSURE TO DDC

5.1 ABSTRACT

Exposure to DDC diet in mice results in primarily biliary injury followed by hepatocyte injury that induces atypical ductular proliferation. This is followed by the appearance of oval cells and eventually the development of fibrosis with chronic injury. We previously reported a potential role for β-catenin in oval cell induction and expansion. After short-term exposure to DDC, β-catenin knockout liver that exhibits deletion of β-catenin in both hepatocytes and cholangiocytes had less A6-positive (oval cell/biliary marker) cells than wild type. To examine the role of β-catenin in response to long-term injury, we exposed WT and KO mice to DDC for 80 and 150 days. Contrary to short-term exposure (<4wk), KO liver harbored significantly more A6 positive atypical ductular and oval cells than WT. This coincided with increased fibrosis, serum bilirubin, and alkaline phosphatase levels in the KO. Interestingly, AST and ALT levels were significantly lower in KO liver at 80 and 150 days upon further analysis, coincident with the observation that β-catenin positive hepatocytes had emerged at both of these times. In order to follow the emergence of β-catenin positive cells, we examined KO liver 30 days after start of DDC feeding and identified 2-8 cell periportal β-catenin positive hepatocyte clusters. These cells

were A6 negative. We also observed tumor formation upon gross examination of KO liver at 150 days, but not in WT. Thus, we observe a β-catenin positive cell population within the KO liver after DDC exposure that undergoes chronic proliferation and expansion, is coincident with improvement in AST and ALT, and eventually results in tumor formation.

5.2 BACKGROUND

The adult hepatic progenitor cell, or oval cell, has proven to be a truly elusive population in the liver. While scarce in healthy liver, a population of cells which emerges in the setting of hepatic injury is clearly documented. These cells typically appear in proximity to the portal triad and have the appearance of small cells with a prominent oval-shaped nucleus and scant cytoplasm. However, populations of cells with somewhat different morphological characteristics and arising from various niches within the liver have been described suggesting significant heterogeneity in the resident progenitor response in liver (148, 219). This has only helped to further complicate the effort to definitively identify and isolate this progenitor population given that the identification of specific markers has been almost impossible in this heterogeneous environment.

Difficulties in definitively identifying the true progenitor cell in the liver have translated to difficulties in elucidating the molecular mechanisms that are involved in the induction and proliferation of the progenitor response. To aid in the quest to identify such molecular mechanisms, various animal models have been developed to study the progenitor response in a controlled environment. One model currently used in mouse is the DDC diet model. It was first described that feeding DDC to mice induced atypical ductular proliferation (ADP) along with periportal inflammation and plugging of the bile ducts with porphyrin crystallization (171).

Ultimately, injury to the biliary epithelium and clogging of the ducts induces biliary stasis and a subsequent rise in serum bilirubin levels. It is believed that hepatic oval cells arise as a response to hepatic injury in this model. A more recent study presented long-term feeding of DDC as a model of xenobiotic-induced cholangiopathy representative of sclerosing cholangitis and biliary cirrhosis (176). DDC feeding led to bile duct injury which induced reactive cholangiocytes and was associated with pericholangitis and periductal fibrosis. Eventually, periductal fibrosis progressed to portal-portal bridging fibrosis and segmental bile duct obstruction occurred. Given that the DDC diet protocol replicates many of the features of chronic cholangiopathies; it is a valuable model for the investigation of the mechanisms of biliary disease and what factors are involved in biliary injury repair.

A role for the Wnt pathway in various stem and progenitor cells is well accepted, as well as clear involvement of the pathway in liver development, growth, and regeneration. Thus, it is possible that the Wnt pathway is involved in the induction of the hepatic progenitor response. Indeed, significant colocalization of the ductular marker A6 and β-catenin occurs after DDC feeding in mice (177). In β-catenin null liver, ADP was significantly blunted indicating that β-catenin may have some role in the induction or proliferation of ductular cells during the response to DDC induced injury. Likewise, another study reported expression of β-catenin in ADP as well as an induction of multiple Wnt ligands after DDC feeding (178). More extensive studies are essential to elucidate what role β-catenin is playing during the response to DDC feeding and by association what potential role it may play in ADP and biliary disease.

To further examine the role for β-catenin in ADP and biliary injury, we subjected wild-type (WT) and β-catenin conditional liver knock-out (KO) mice to long-term feeding of the DDC diet. These KO mice lack β-catenin in both hepatocytes and biliary epithelial cells. Animals were

exposed to the diet for 50-150 days and examined histologically and biochemically for the ductular response and liver injury. Interestingly, we found that in the absence of β-catenin, a compensatory hyperproliferation of ductular cells occurs in the long-term, along with the development of significant hepatic fibrosis. As early as 30 days of DDC feeding, there were emergence of isolated β-catenin positive periportal hepatocyte clusters (2-8 cells) that over a period of time repopulate the KO liver. These cells show ongoing proliferation and eventually led to development of hepatic tumors by 150 days.

5.3 RESULTS

5.3.1 Increased ductular proliferation in β-catenin KO after long-term DDC feeding

Previous studies have shown a blunted ADP in β-catenin KO after DDC feeding for 2 and 3.5 weeks (177). We sought to expand on this finding by testing long-term DDC feeding in the same mouse model. Wild-type (WT) and KO mice were placed on DDC diet for time periods ranging from 80 to 150 days to examine long-term ductular proliferation and hepatic injury. H&E staining, along with immunofluorescence was performed to survey ADP during this time. Interestingly, H&E staining showed a progressive increase in ADP that occurred in KO liver when compared to WT at 80 and 150 days of DDC feeding (Fig. 15a). Immunofluorescence for A6, a ductular/oval cell marker, was performed to verify and quantify this finding. Indeed, significantly more A6 positive cells are found in KO liver at 80 and 150 days of DDC feeding (Fig. 15b, c). If this data is combined with previous published data suggesting a blunted initiation

of ADP in KO, there is a compensatory response that occurs between 30 and 80 days where the number of ductular cells increases at a faster rate than in WT liver.

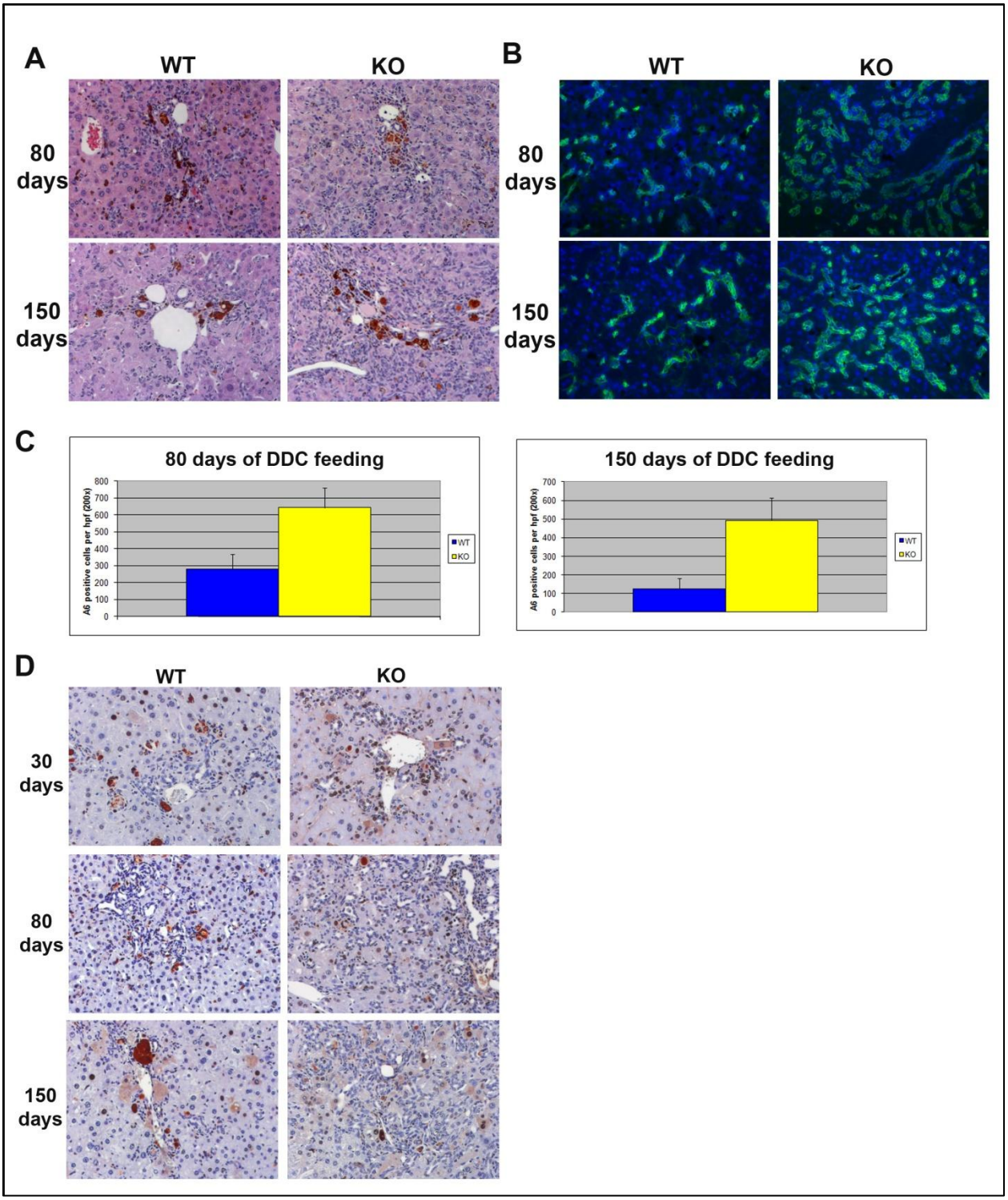


Figure 15: Increased ADP in KO after long-term DDC.

(A) Representative photomicrographs of H&E staining of WT and KO livers after 80 and 150 days of DDC diet feeding. **(B)** Representative photomicrographs of immunofluorescence for A6 (green) in WT and KO livers after 80 and 150 days of DDC diet feeding. Counterstain is with DAPI (blue, nuclei). **(C)** Quantification of A6 positive cells in WT and KO livers at 80 and 150 days of DDC diet feeding shows an increase in KO vs. WT. **(D)** Representative photomicrographs for PCNA IHC in WT and KO liver after 30, 80, and 150 days of DDC diet feeding. All images at 200x magnification. *-p<.01

To examine whether an increase in proliferation of ductular cells was the cause for this compensatory response, PCNA staining was performed on samples at 30, 80, and 150 days after DDC feeding. An increase in PCNA positive ductular cells is evident in KO compared to WT as early as 30 days and still observed at 80 days of DDC diet feeding (Fig. 15d). Proliferation of atypical ductular cells is fairly evident in both KO and WT at 150 days. These findings are in accordance with the increase in A6 positive cells beyond 30 days of DDC feeding in the KO, both of which suggest that a compensatory hyperproliferation of ductular cells occurs in KO after long-term DDC feeding.

5.3.2 Increased serum bilirubin, fibrosis, and tumorigenesis in KO after long-term DDC

To monitor hepatic injury after long-term DDC feeding, serum analysis was performed for markers of hepatic injury (AST and ALT) and cholestasis (bilirubin and alkaline phosphatase (ALP)). Levels of AST were modestly higher at 150 days of DDC feeding in WT compared to KO, whereas ALT levels were higher in WT at 80 and 150 days (Tables 8&9). This finding was surprising given that we expected KO to be more susceptible to hepatocyte injury. On other hand, serum bilirubin levels were significantly higher in the KO at 80 days with a trend towards

an increase in KO at 150 days. Additionally, levels of alkaline phosphatase were higher in KO at 150 days of DDC feeding. These findings indicate that cholestasis after long-term DDC feeding is worse in the KO than what is observed in WT.

Table 8: Serum biochemistry after 80 days of DDC diet feeding (*p<0.05)

Serum Biochemistry		
Total Bilirubin	***WT***	***KO***
1	0.4	11.8
2	0.3	11.1
3	0.8	1.9
4	5.6	5.4
5	0.4	
6	0.1	
7	0.1	
8	0.2	
Average	**1.07**	**6.133**
Alkaline Phosphatase	***WT***	***KO***
1	254	647
2	315	518
3	435	319
4	493	209
5	395	272
6	298	
7	264	
8	199	
Average	**331.625**	**393**

AST	***WT***	***KO***
1	268	486
2	331	349
3	604	173
4	398	334
5	231	261
6	298	
7	443	
8	287	
Average	**355**	**320.6**
ALT	***WT***	***KO***
1	634	408
2	713	251
3	914	243
4	797	366
5	526	428
6	509	
7	1033	
8	617	
Average	**717.875**	**339.2***

Table 9: Serum biochemistry after 150 days of DDC diet feeding (*p<0.05)

Serum Biochemistry		
Total Bilirubin	***WT***	***KO***
1	0.3	0.7
2	0.3	1.6
3	0.4	1.2
4	0.4	11.8
5	0.4	1.1
6	0.2	1.1
7	1.0	0.8
Average	**0.45**	**2.933**
Alkaline Phosphatase	***WT***	***KO***
1	173	524
2	106	367
3	380	456
4	197	549
5	168	416
Average	**204.8**	**462.4***
AST	***WT***	***KO***
1	556	426
2	343	334
3	952	462
4	732	751
5	727	495
6	996	263
7	1186	
Average	**717.6667**	**455.1667***

ALT	***WT***	***KO***
1	827	509
2	434	367
3	1215	574
4	908	480
5	882	497
6	1476	411
7	1566	
Average	**1044**	**473***

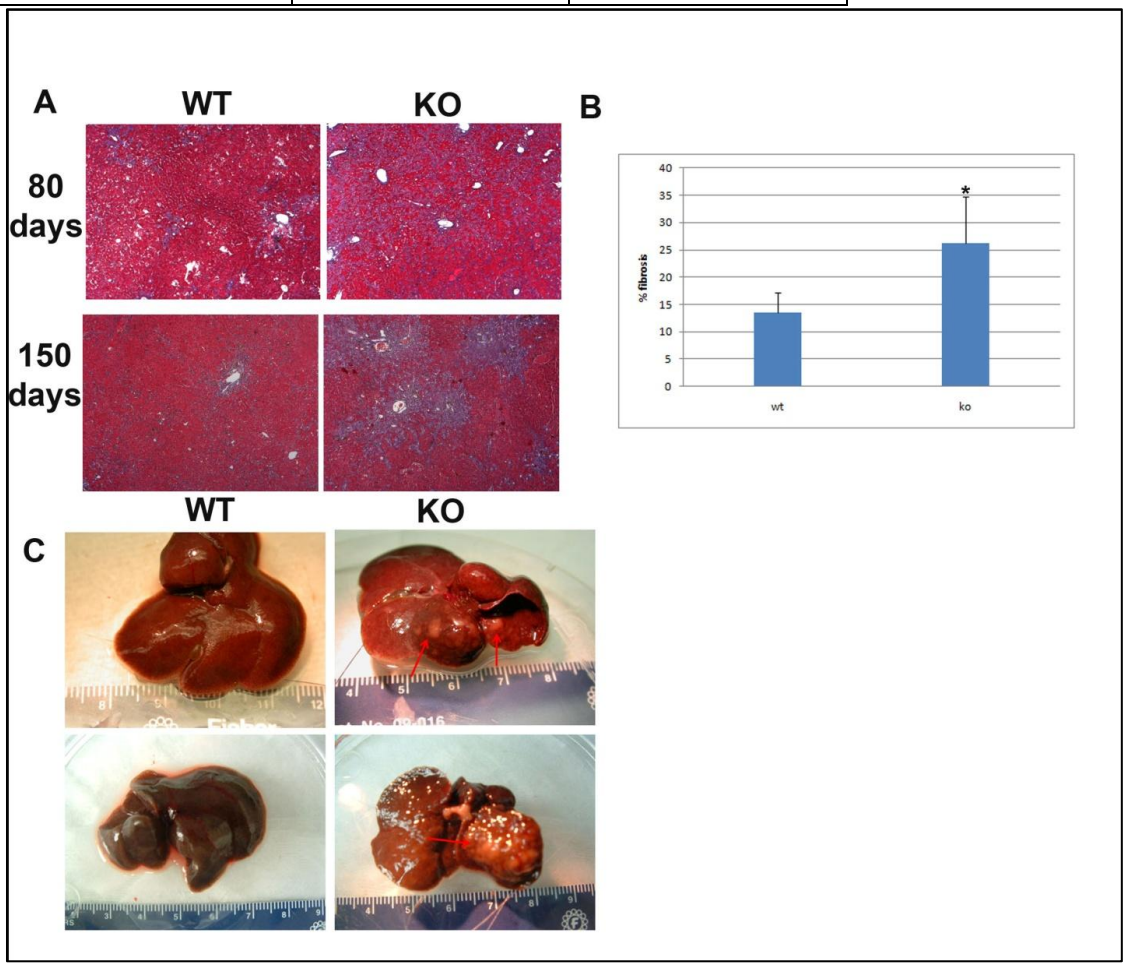


Figure 16: Increase in hepatic fibrosis, serum bilirubin, and tumor development in KO after long-term DDC feeding.

(A) Representative photomicrographs for trichrome (fibrosis, blue) of liver from WT and KO after 80 and 150 days of feeding with DDC diet. Images at 50x magnification. **(B)** Quantification of % fibrosis at 150 days of feeding with DDC diet shows an increase in KO compared to WT. **(C)** Appearance of tumors (arrows) in KO liver after 150 days of DDC diet feeding. Tumors are not observed in WT. *$p<0.05$

It has been previously reported that DDC feeding induces activation of stellate cells which results in periportal fibrosis which eventually progresses to bridging fibrosis and cirrhosis in the mouse liver (176). We performed trichrome staining to analyze the amount of fibrosis in our study. Not surprisingly, KO liver showed more significant fibrosis after 80 and 150 days of DDC feeding (Fig. 16a). Overall, percent fibrosis was twice as much in KO when compared to WT at 150 days (Fig. 16a&b). At 150 days, bridging fibrosis was evident in the KO liver suggestive of significant progression of disease in these animals. We also observed the formation of tumors in 6 out 7 KO after 150 days of DDC diet feeding, but not in the WT (Fig. 16c).

5.3.3 Progressive repopulation of KO liver hepatocyte population with β-catenin positive cells after long-term DDC feeding

Given the presence of increased cell proliferation and tumor development in KO mice, we sought to verify any β-catenin expression in KO. Western blot analysis was performed on livers from KO and WT mice after 150 days of DDC feeding to examine for β-catenin expression. Surprisingly, expression levels of β-catenin in the KO at 150 days of DDC were similar to that observed in WT (Fig. 17a). To validate this finding, immunohistochemical analysis for β-catenin was performed. At 150 days, β-catenin is expressed throughout the entire liver tissue examined (Fig. 17b). Interestingly, at 80 days of DDC feeding, small clusters of β-catenin expressing

hepatocytes can be observed periportally and are surrounded by otherwise β-catenin negative tissue confirming that these animals are indeed KO's. Following this back further to only 30 days after DDC feeding shows that only a few β-catenin positive hepatocytes are present in the periportal region (Fig. 17b). These findings are suggestive of repopulation of the KO liver by some β-catenin positive source as there is a clear time course to the appearance of β-catenin positive cells.

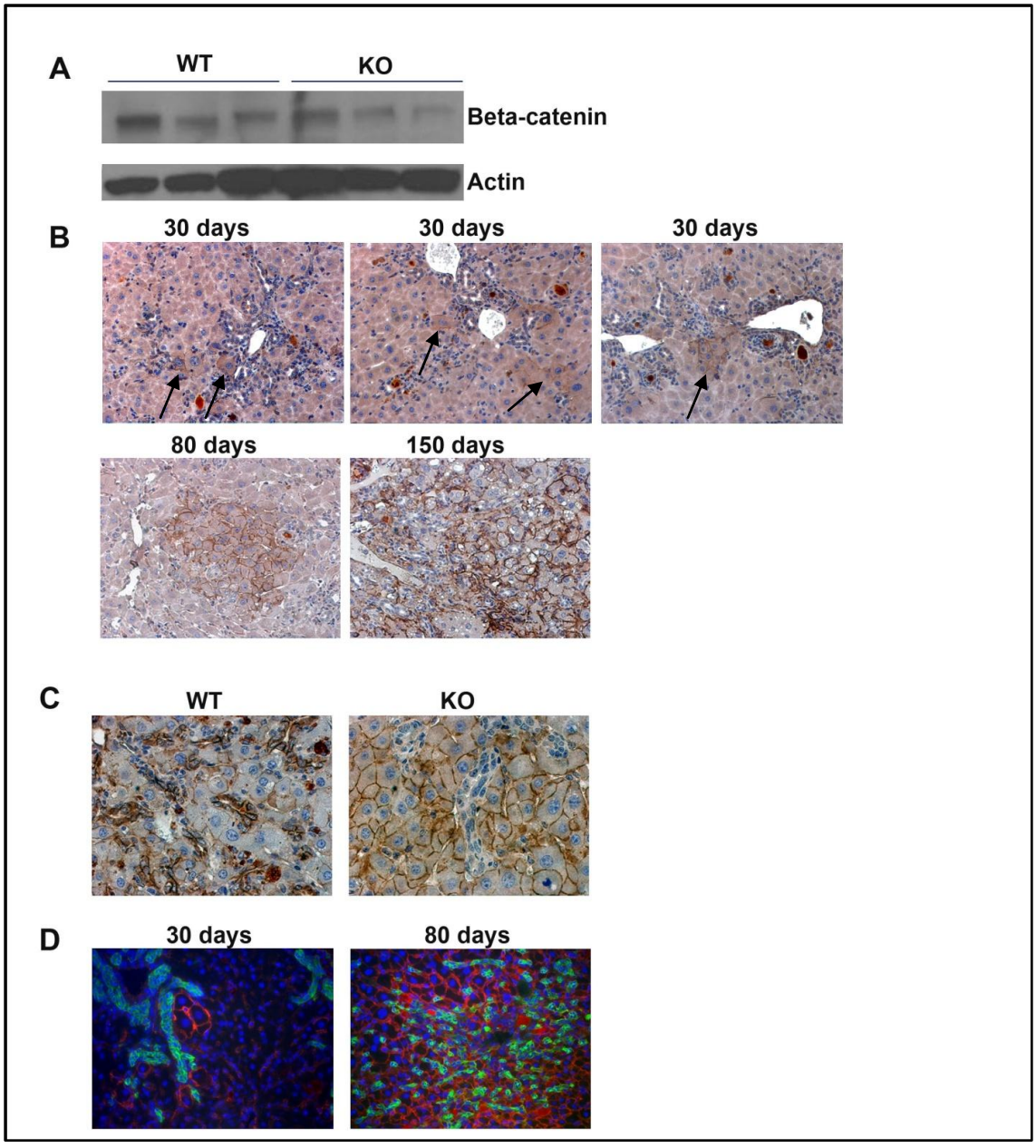


Figure 17: Repopulation of KO liver with β-catenin positive hepatocytes over the course of long-term feeding of DDC diet.

(A) Western blot showing total protein levels of β-catenin in WT and KO liver after 150 days of feeding of DDC diet. Actin was used as a loading control. **(B)** Representative photomicrographs for IHC for β-catenin in KO liver at 30, 80, and 150 days of feeding of DDC diet showing progressive repopulation of KO liver with β-catenin positive hepatocytes. Images at 200x magnification. **(C)** Representative photomicrographs of IHC for β-catenin after 80 days of DDC diet feeding in WT and KO showing that expression of β-catenin in atypical ductular cells does not occur in KO even within clusters of β-catenin positive hepatocytes. Images at 400x magnification. **(D)** Representative photomicrographs of immunofluorescence for A6 (green) and β-catenin (Red) in KO liver at 30 and 80 days of DDC diet feeding showing no colocalization. Images at 200x magnification.

Interestingly, expression of β-catenin in the KO liver after DDC seems to be restricted to hepatocytes as biliary epithelial cells and atypical ductular proliferating cells are negative for β-catenin expression (Fig. 17c). This absence of expression in ductular cells is true for portal areas surrounded by β-catenin negative hepatocytes as well as within clusters of β-catenin positive cells. Conversely, β-catenin expression is quite abundant in both oval cells and ADP in the WT after DDC. To confirm absence of β-catenin expression in ductular cells in KO liver, co-localization studies were performed for A6 and β-catenin which shows that A6 positive cells in KO are β-catenin negative (Fig. 17d). This suggests that cholangiocytes and oval cells are not the source of β-catenin hepatocytes appearing in the KO liver after long-term DDC feeding and hence the repopulation of KO liver by β-catenin positive hepatocytes is through a novel periportal population of cells.

5.4 DISCUSSION

We previously reported that β-catenin KO mice show a blunted ADP after DDC feeding for 2 and 3.5 weeks (177). However, in this study we report that atypical ductular proliferation takes off dramatically in KO after 3.5 weeks and is significantly increased over WT from 80 to 150 days of DDC feeding. Additionally we show that KO exhibit higher levels of serum bilirubin, alkaline phosphatase, and hepatic fibrosis but lower AST and ALT after long-term DDC feeding. Concomitantly, the KO liver experiences a gradual repopulation by β-catenin positive hepatocytes starting around 30 days after DDC feeding and reaches close to 100% of hepatocytes by 150 days, whereas ADP and cholangiocytes are negative for β-catenin. We also find that most of the KO animals have developed tumors in the liver, a phenomenon not observed in the WT.

The chief observation in this study consisted of repopulation of the β-catenin KO liver with a de-novo population of β-catenin positive hepatocytes that appear to have originated periportally. It is relevant to note that none of these single isolated cells were positive for oval cell markers such as A6, and all biliary epithelial cells were negative for β-catenin suggesting that the origin of these cells is not from the bile ducts. However, once β-catenin positive hepatocytes emerge, they have distinct proliferation and survival advantages and repopulate the entire KO liver within several weeks. Interestingly, hepatocyte injury represented by levels of AST and ALT was reduced over time presumably due to repopulation by β-catenin positive hepatocytes. β-catenin is known to regulate expression of anti-oxidant and xenobiotic metabolism associated genes.

What is the origin of the A6-negative, β-catenin-positive cells observed periportally at 30 days after DDC exposure? One possibility is that the de-novo population is a stem cell population. Several stem cell populations have been reported, albeit their interrelationship to

each other and complete characterization remains debatable. It will be essential to examine the periportal β-catenin-positive cells at the earliest stages of their appearance for expression of various markers such as Trop-2 (oval cell), α-smooth muscle actin (stellate cell), CD14 (Kuppfer cell), Sca1 (hematopoietic stem cell), and α-fetoprotein (immature hepatocyte), as well as other markers. A caveat, to the stem cell origin of β-catenin-positive population is that after the de-novo population appears and becomes hepatocytes, they continue to retain β-catenin expression several weeks later. Since albumin expression would be turned on if these cells were truly mature hepatocytes, it should activate albumin-cre transgene and thus lead to deletion of β-catenin. Thus, it appears that β-catenin positive hepatocytes that repopulate the KO liver are predominantly in the immature form even at late stages of DDC exposure. In support of this hypothesis, β-catenin KO liver after 150 days of DDC exposure show a significant decrease in expression of markers of hepatocyte maturation (CEBPα, GS, Cyp2E1). It is likely that when subsets of these immature hepatocytes undergo maturation, β-catenin deletion ensues and these cells succumb to the ongoing injury induced by continuous DDC exposure and thus are undetectable at later stages. Role of β-catenin in hepatocyte survival is unquestionable (78, 84). The predominance of immaturity of the β-catenin-positive hepatocytes in KO livers even at late stages of DDC exposure is also supported by the presence of pronounced cell proliferation. Ultimately the β-catenin positive immature hepatocytes continually proliferate and lead to tumor formation in 6/7 KO mice exposed to DDC for 150 days.

Intriguingly, no ADP, oval cells, or cholangiocytes were positive for β-catenin suggesting that no derivation of newly formed ducts from β-catenin positive hepatocytes occurred in contrast to what we showed in β-catenin over expressing TG mouse liver exposed to DDC. It is relevant to remind that transdifferentiation of hepatocytes to biliary epithelial cells was

demonstrated previously as well. A role for β-catenin in biliary specification has been shown by us and others, both in vitro and in vivo. While the exact mechanism of this remains to be investigated, loss of β-catenin has been shown to lead to loss of CK19 positive cells via lack of biliary commitment of hepatoblasts in embryonic liver cultures. Conversely, activation of β-catenin by exogenous Wnt3a led to differentiation of hepatoblasts to cholangiocytes (86). Thus the current model shows that the de-novo population of β-catenin positive cells is predominantly of hepatocyte lineage only. It is interesting to hypothesize that this positive cell is indeed derived from a novel bipotential stem cell of non-hepatobiliary lineage at its inception. However, lack of β-catenin in such a cell during its most undifferentiated state disallows its commitment to biliary cell type but does not impede its differentiation to hepatocyte and hence the observed phenotype as has been shown in liver development studies. At the same time, this observation of absent β-catenin in ADP and cholangiocytes at all times after DDC exposure in the KO, precludes derivation of the earliest β-catenin-positive cells or stem cells from β-catenin negative ADP or cholangiocytes. The end result of enhanced albeit β-catenin-negative ADP in KO exposed to DDC is a pronounced effect on biliary dysfunction as shown by increased bilirubin and alkaline phosphatase levels when compared to WT. Also, there is an increase in periportal fibrosis and inflammation due to ongoing lack of optimal repair in KO when compared to WT mice, which have normal expression of β-catenin. This clearly demonstrates a lack of adequate reparative response in the absence of β-catenin in ADP and cholangiocytes further supporting an important role of Wnt/β-catenin signaling in bile duct homeostasis. Indeed, β-catenin over expressing mice exposed to DDC demonstrate a significant improvement in intrahepatic cholestasis as demonstrated in the previous chapter.

5.5 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Findings

- Compensatory hyperproliferation of atypical ductular cells after long-term (50-150 days) feeding of DDC diet in β-catenin KO liver.
- KO liver is repopulated by β-catenin positive hepatocytes over the course of long-term feeding of DDC diet; however, these cells continue to show an immature and proliferative phenotype which improved levels of AST and ALT but eventually yielded tumors in the KO liver.
- ADP and cholangiocytes continue to be β-catenin negative and KO show increased serum bilirubin, alkaline phosphatase, and hepatic fibrosis in KO compared to WT after long-term feeding of DDC diet supporting an important role for β-catenin in biliary differentiation and homeostasis.

Future Directions

- Perform additional marker analysis to characterize β-catenin positive cells at early stages in KO after DDC exposure.
- Perform lineage tracing studies to identify the source of β-catenin positive hepatocytes in KO liver after DDC exposure.
- Examine other models of biliary injury in the β-catenin KO mice to see if those models induce repopulation of the liver with β-catenin positive hepatocytes as well.

6.0 PEGYLATED INTERFERON TARGETS WNT SIGNALING BY INDUCING NUCLEAR EXPORT OF β-CATENIN

6.1 ABSTRACT

Pegylated-Interferon-α2a (Peg-IFN) is a first line therapy for Hepatitis C virus (HCV) patients and also impacts recurrence of hepatocellular carcinoma (HCC) in these patients. Wnt pathway activation due to β-catenin gene mutations contributes to the development of a significant subset of HCC. Here, we explored the effect of Peg-IFN on Wnt/β-catenin signaling *in vitro* and *in vivo*. Transgenic mice over expressing a stabilized form of β-catenin were exposed to diethylnitrosamine and treated with Peg-IFN to examine effect on the Wnt pathway. Human hepatoma cell lines (Hep3B, HepG2, Huh-7, & Snu-449) were treated with Peg-IFN to measure the effect of treatment on Wnt pathway and elucidate the mechanism. *In vivo*, we observed a decrease in nuclear levels of β-catenin and cyclin D1, which was coincident with a decrease in proliferation. *In vitro*, 3 or 4 doses of Peg-IFN decreased transcriptional activity of β-catenin/Tcf and did so independent of JAK/Stat signaling. Real-time PCR showed an increase in the expression of a nuclear export factor RanBP3 in all 3-cell lines. A concomitant increase in RanBP3 protein was also evident. Its role in negatively regulating Wnt signaling was confirmed as RanBP3 siRNA knockdown reversed the Peg-IFN-induced decrease in TOPflash assay. Reanalysis of *in vivo* study detected an increase in RanBP3 protein levels in response to Peg-IFN

treatment. Furthermore, increased association of RanBP3 and β-catenin was established in Peg-IFN treated liver through co-immunoprecipitation studies. Peg-IFN inhibits β-catenin signaling through upregulation of RanBP3 which associated with β-catenin resulting in nuclear export. This may be a contributory mechanism of delayed HCC and improved survival in Peg-IFN treated HCV patients. This observation might have chemo preventive or chemotherapeutic implications in tumors with aberrant Wnt/β-catenin activation.

6.2 BACKGROUND

Interferons (IFN) are a family of glycoprotein cytokines that were first described for their antiviral activity (220). Indeed, IFNs are currently a first line therapy for reducing viral load in patients infected with hepatitis C virus. Studies have also identified the role of these cytokines in controlling cell proliferation and differentiation (221, 222). In fact, IFNs have proven useful in inhibiting proliferation of tumor cells in animals (223). Such anti-proliferative activity makes IFN an intriguing option for chemoprevention and chemotherapy.

One of the long-term consequences of HCV infection is the development of hepatocellular carcinoma (HCC). Various studies have reported range of effects of IFN therapy on HCC in HCV patients ranging from effectively preventing the development of HCC to only reducing the late recurrence of HCC in HCV-infected patients (138, 224, 225). Interestingly, even patients who were classified as not having a response to therapy showed a decrease in development of HCC. Furthermore, several case reports have reported successful treatment of HCC with IFN-α as part of the chemotherapeutic regimen (226-229). Thus, it is conceivable that IFN-α may have anti-tumor effects beyond decreasing the viral load in these patients. In fact,

IFN-α has also shown to promote apoptosis in human hepatoma cells in the absence of HCV infection (230).

A molecular mechanism for IFN-α's anti-tumor effects is yet to be elucidated. It was previously reported that β-catenin mutations occur in approximately 40% of HCV patients who develop HCC (137). Additionally, HCV nonstructural NS5A protein activity ultimately leads to phosphorylation and inactivation of GSK3β, one of the key players in the degradation of β-catenin (231). Such activity leads to accumulation of β-catenin in the cytoplasm and nucleus with subsequent transcriptional activation of Wnt pathway targets. These findings implicate the Wnt/β-catenin pathway as a culprit in HCV-associated hepatocarcinogenesis.

Given the likely role of β-catenin in HCV-associated HCC and the anti-tumor effects of pegylated interferon-α2a (Peg-IFN), we investigated the hypothesis that peg-IFN could negatively regulate Wnt/β-catenin signaling. We report here evidence from studies on human hepatoma cell lines, as well from *in vivo* experiments with serine-45 mutated-β-catenin transgenic mice, that Peg-IFN can effectively target the Wnt pathway. Intriguingly, this effect appears to be mediated by upregulation of a nuclear export factor, RanBP3. Ultimately, the results presented support the efficacy of utilizing Peg-IFN to inhibit the Wnt/β-catenin pathway in hepatocellular carcinoma.

6.3 RESULTS

6.3.1 Peg-IFN decreases β-catenin activity and proliferation in vivo

To initially examine the effect of Peg-IFN on the Wnt signaling pathway, we treated TG mice over expressing serine-45 mutant β-catenin (TG) in liver and exposed to diethylnitrosamine (DEN), a hepatocarcinogen commonly used in mice. Briefly, mice received a single IP injection of DEN at day 15 after birth and were initiated on saline or Peg-IFN treatment 10 weeks later. Treatments were administered via subcutaneous injection once weekly for six weeks. Two dosages of Peg-IFN, 7,000U and 70,000U, were examined which were used previously in mouse models of carcinogenesis (232, 233). Treatment at both doses resulted in a decrease in nuclear levels of β-catenin, an indicator of activation of this pathway, and cyclin D1, a target of the Wnt pathway (Fig. 18a). Glutamine synthetase, a specific target of the Wnt pathway, was also modestly decreased after treatment with Peg-IFN. To assess any functional effect of Peg-IFN treatment on levels of hepatocyte proliferation, immunohistochemistry for PCNA was performed. A decrease in the number of PCNA positive hepatocytes was evident after Peg-IFN treatment (Fig. 18b), which was also statistically significant (Fig. 18c).

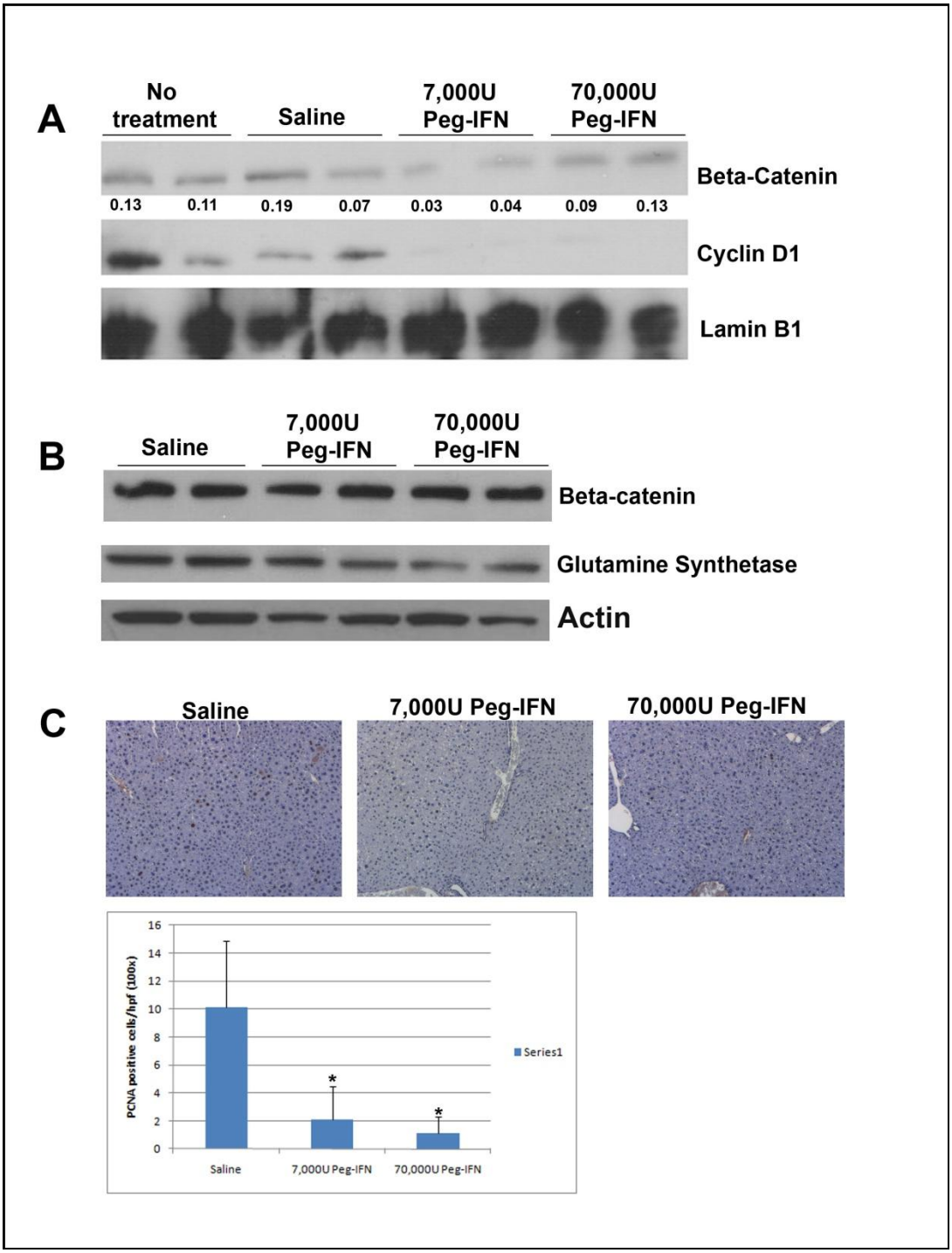


Figure 18: Peg-IFN decreases β-catenin activity in vivo.

(A) Immunoblotting shows decreased levels of nuclear β-catenin and cyclin D1 after treatment with Peg-IFN (7,000U or 70,000U) weekly for 6 weeks. **(B)** Immunoblotting shows a modes decrease in GS after treatment with Peg-IFN weekly for 6 weeks. **(C) Top:** IHC for PCNA in liver of animals treated with saline or Peg-IFN. **Bottom:**

Quantification shows a significant decrease in the number of PCNA positive hepatocytes per high power field (200x) in the liver of animals treated with Peg-IFN at either dose vs. saline (*$p<0.05$).

6.3.2 Peg-IFN decreases β-catenin/Tcf-mediated transcriptional activity in human hepatoma cells

Next, we utilized the TOPFlash luciferase reporter assay to directly measure β-catenin/Tcf-dependent transcriptional activation in Peg-IFN-treated HepG2 cells. HepG2 cells carry a deletion mutation in exon 3 of β-catenin rendering it stable and constitutively active, thus baseline β-catenin/Tcf-mediated transcriptional activity is high in these cells (123). We anticipated that treatment of HepG2 cells with Peg-IFN would result in a decrease in TOPFlash luciferase activity. Indeed, treatment with 3 doses of Peg-IFN resulted in a 37% decrease in reporter activity (Fig. 19A, $p<0.05$). Moreover, four doses of Peg-IFN resulted in a greater (50%) decrease in reporter activity ($p<0.005$). We also tested the effect of Peg-IFN treatment on a hepatoma cell line expressing wild-type β-catenin, the Hep3B cell line. It only took one dose of Peg-IFN to significantly decrease TOPFlash reporter activity (Fig. 19b). After treatment with LiCl, a known activator of the Wnt pathway via inhibition of GSK3β, TOPFlash reporter activity increases over control. Treatment with Peg-IFN also significantly decreased reporter activity after stimulation with LiCl further showing that Peg-IFN can negatively regulate β-catenin when it is in an activated state (Fig. 19b). Thus, we definitively show that Peg-IFN is capable of decreasing activation and subsequent downstream transcriptional activity of the Wnt/β-catenin pathway in hepatoma cells.

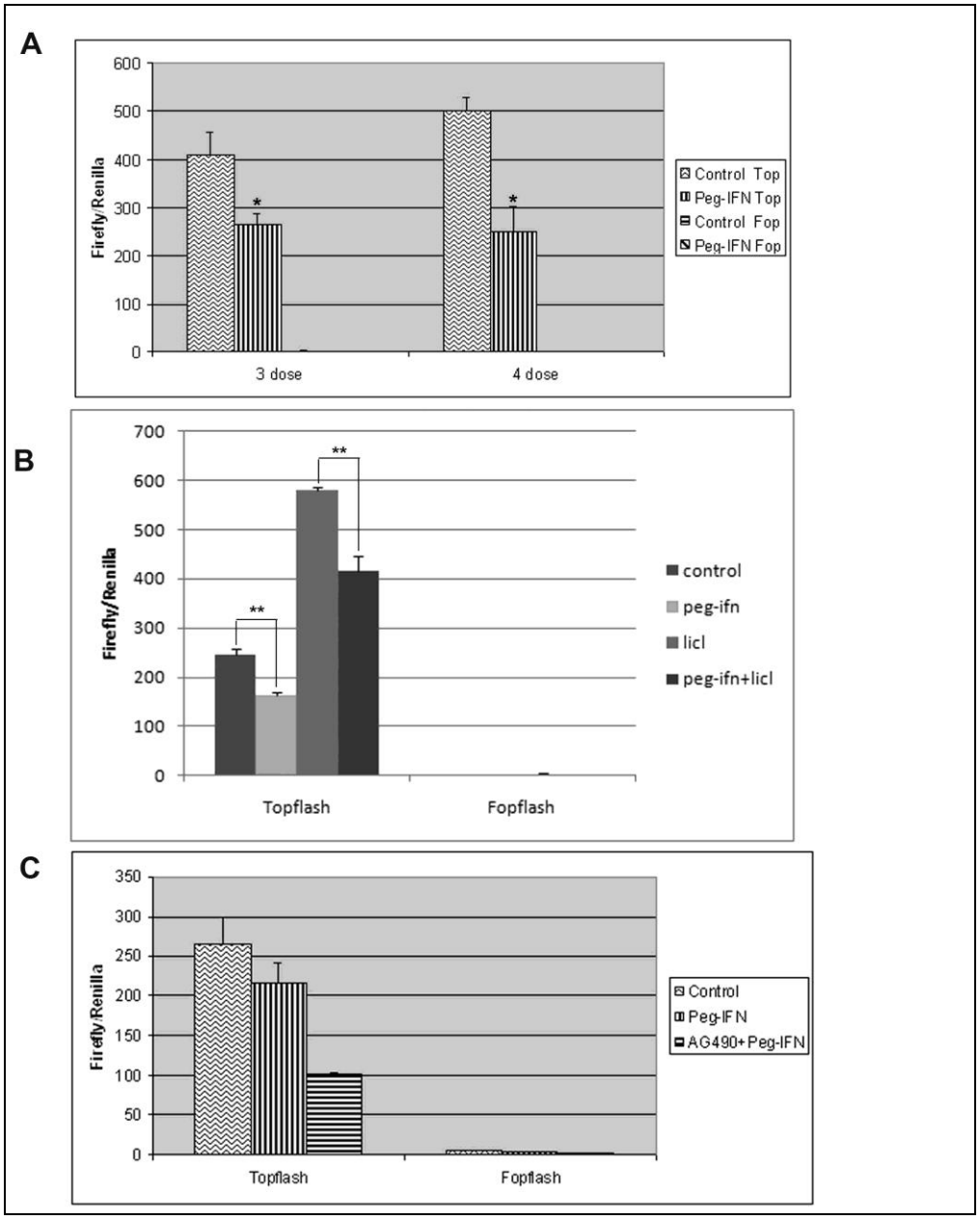


Figure 19: Peg-IFN decreases β-catenin activity in human hepatoma cell lines.

(A) Relative luciferase activity in HepG2 cells after 3 or 4 dose Peg-IFN treatment. **(B)** Relative luciferase activity in Hep3B cells decreases after Peg-IFN treatment before and after stimulation of the Wnt pathway with LiCl. **(C)** Inhibition of JAK/Stat pathway with AG490 does not reverse the effect of Peg-IFN on Topflash in HepG2 cells indicating that this effect is independent of the JAK/Stat pathway. All experiments were done in triplicate and averaged with standard deviation shown. (*$p<0.05$, **$p<0.01$)

6.3.3 Peg-IFN effect on Wnt Pathway independent of Jak/Stat signaling

Given that interferon cytokines typically signal via the Jak/Stat pathway (234), we wanted to explore the possibility that the anti-β-catenin effect of Peg-IFN's maybe dependent on these downstream effectors. To investigate, we simultaneously treated HepG2 cells with Peg-IFN and a chemical inhibitor of JAK, AG490, with the expectation that transcriptional activity would return to normal levels if the anti-β-catenin effect of Peg-IFN was indeed JAK/Stat-dependent. On the contrary, we observed that inhibition of the JAK/Stat pathway actually enhanced the anti-β-catenin activity following Peg-IFN treatment (Fig. 19c), thus Peg-IFN appears to inhibit Wnt signaling independent of JAK/Stat signaling.

6.3.4 Peg-IFN increases transcription of Dkk-1 and RanBP3

After identifying that Peg-IFN was acting on the Wnt pathway independent of JAK/Stat, we decided to explore several other factors known to negatively regulate Wnt/β-catenin signaling. One well-characterized inhibitor of Wnt signaling is Dikkopf-1 (Dkk-1), which was previously shown to be upregulated in cells treated with IFN-α (235, 236). Indeed we observed that gene expression of Dkk-1 was upregulated in both Hep3B (16-fold) and HepG2 cells (4-fold) (Fig. 20a-b). While this finding is likely to be relevant in cell lines which contain wild type β-catenin, such as Hep3B, the typical manner in which Dkk-1 downregulates Wnt signaling, i.e. through prevention of Wnt- Frizzled-LRP5/6 complex (237), would likely be ineffective in tumor cells that have downstream defect resulting in Wnt activation such as in HepG2 cells, which harbor mutation in the β-catenin gene. Given this, we explored any changes in another known mediator of Wnt signaling, the Ran Binding Protein 3 (RanBP3). It was previously reported that RanBP3

is involved in the nuclear export of β-catenin and, importantly, is capable of acting on both wild type and deletion mutant forms of β-catenin (30). Interestingly, we observed a multi-fold increase in levels of gene expression of RanBP3 in Hep3B, HepG2, and Snu-449 cells after peg-IFN treatment (Fig. 20a-c).

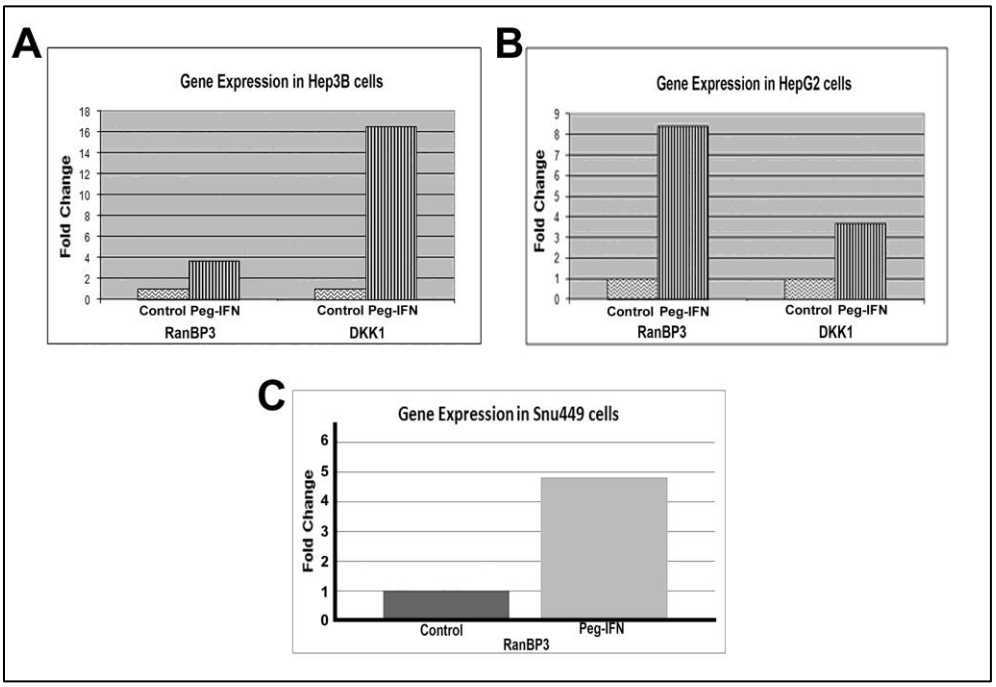


Figure 20: Peg-IFN increases levels of Dkk-1 and RanBP3.

(A) Real-time PCR showing increase in expression of Dkk-1 and RanBP3 in Hep3B cells after Peg-IFN. Values normalized to control and presented as fold-change. (**B)** Real-time PCR showing increase in expression of Dkk-1 and RanBP3 in HepG2 cells after Peg-IFN. Values normalized to control and presented as fold-change. **(C)** Real-time PCR showing increase in RanBP3 in Snu-449 cells after Peg-IFN. Values normalized to control and presented as fold-change.

6.3.5 Increased levels of RanBP3 protein after Peg-IFN treatment in vitro and in vivo

To determine if an increase in gene expression translated to an increase in protein levels we measured levels of RanBP3 protein in human hepatoma cells treated with Peg-IFN. Indeed, a

dose-dependent increase in levels of RanBP3 occurred after treatment with Peg-IFN (Fig. 21a). As mentioned previously, RanBP3 binds to β-catenin and induces its nuclear export (30). Indeed, we observe an increase in association between RanBP3 and β-catenin in HepG2 cells after Peg-IFN treatment (Fig. 21b). To determine if RanBP3 was also increased in vivo we examined levels of RanBP3 protein in TG liver following treatment with Peg-IFN which showed a clear increase in RanBP3 (Fig. 21c). To further explore if this increase in protein levels had any consequence on Wnt signaling in vivo, we examined association of RanBP3 with β-catenin. We observed a dramatic increase in association between these two proteins after Peg-IFN treatment further strengthening the claim that nuclear export of β-catenin by RanBP3 as a chief mechanism of inhibition of β-catenin activity (Fig. 21d).

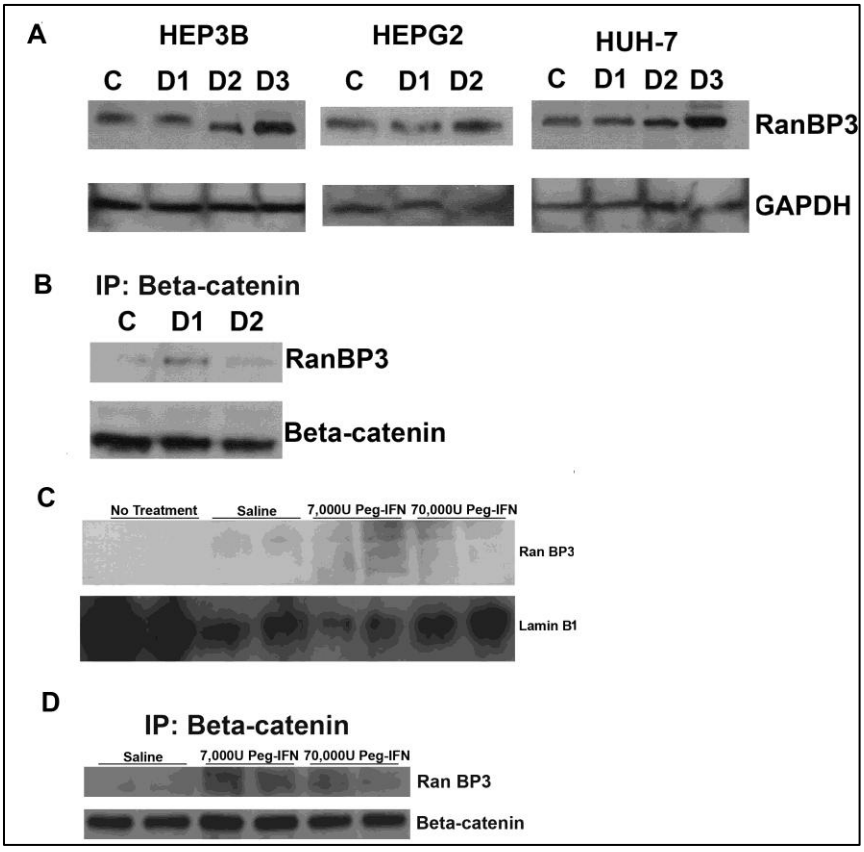


Figure 21: Increased protein expression of RanBP3 and association with β-catenin after treatment with Peg-IFN.

(A) Protein expression for RanBP3 in human hepatoma cell lines (Hep3B, HepG2, and Huh-7) after treatment with 1, 2, or 3 doses of Peg-IFN (100 U/ml). GAPDH is loading control. **(B)** Co-immunoprecipitation of protein lysates from HepG2 cells after 1 or 2 doses of Peg-IFN showing increased association of RanBP3 with β-catenin. **(C)** Protein expression of RanBP3 in nuclear fraction from livers of TG mice after no treatment, saline treatment, or treatment with Peg-IFN (7,000U or 70,000U). LaminB1 is loading control. **(D)** Co-immunoprecipitation of protein lysates from livers of TG mice after saline treatment or treatment with Peg-IFN (7,000U or 70,000) showing increased association between RanBP3 and β-catenin after Peg-IFN treatment.

6.3.6 RanBP3 acts downstream of Peg-IFN to negatively regulate Wnt pathway

To verify the negative regulatory effect of RanBP3 on the canonical Wnt signaling in human hepatoma cells, we over expressed RanBP3 in HepG2 cells and measured the effect on pathway activity by the TOPFlash assay. Indeed, HepG2 cells transfected with a RanBP3 over expression vector exhibited a significant decrease in reporter activity much like was observed following treatment with Peg-IFN (Fig. 22a). To definitively confirm that Peg-IFN's effect on the Wnt pathway was ultimately via increased RanBP3, we simultaneously performed Peg-IFN treatment and siRNA knockdown of RanBP3 (Fig. 22b, upper panel). After treatment with three doses of Peg-IFN along with knockdown of RanBP3, we observed an increase in beta-catenin/Tcf mediated transcriptional activity suggesting that RanBP3 was necessary for the negative effects of the Peg-IFN on Wnt/β-catenin pathway (Fig. 22b, lower panel). Thus, the negative regulatory effect of Peg-IFN on the Wnt pathway is at least partly explained through the upregulation of RanBP3.

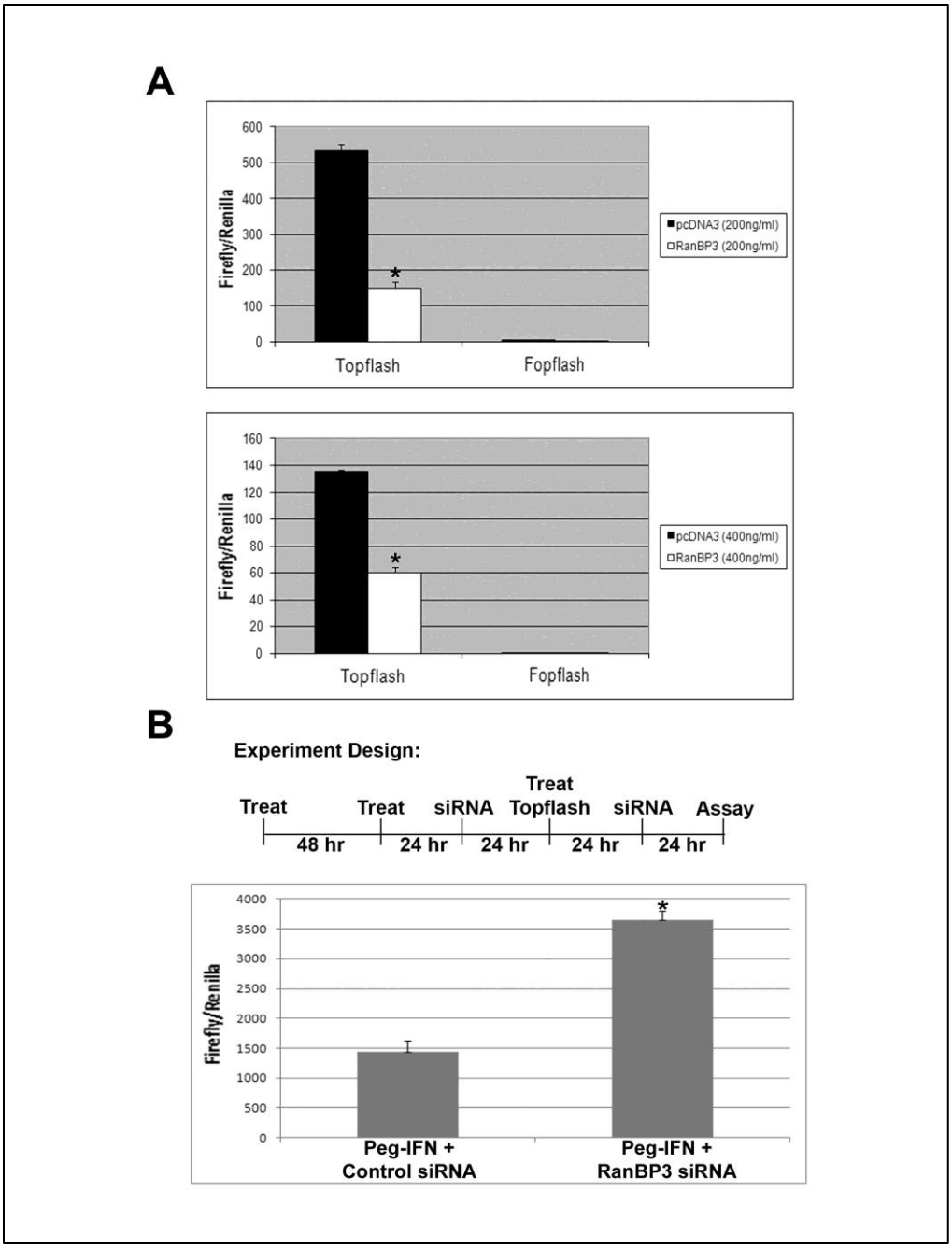


Figure 22: Negative effect on Wnt pathway after Peg-IFN treatment is via upregulation of RanBP3.

(A) TOPFlash luciferase assay showing a decrease in reporter activity after over expression of RanBP3 at 200ng/ml (Top) and 400ng/ml (Bottom) in HepG2 cells. (**B)** Topflash luciferase assay showing a recovery of reporter activity after siRNA mediated downregulation of RanBP3 simultaneously with Peg-IFN treatment. Experimental design is outlined above the graph. (*$p<0.01$)

6.4 DISCUSSION

While an important player in liver development and growth, the Wnt/β-catenin pathway has been implicated as an oncogenic factor in hepatocarcinogenesis. Our laboratory has performed proof-of-principle studies demonstrating decreased survival and proliferation of human liver tumor cells secondary to β-catenin suppression (144, 193). Successful targeting of the Wnt/β-catenin pathway via drug therapy as a treatment strategy for HCC patients is thus relevant and of high translational significance (199, 238). This is also true for many cases of HCC which exhibit β-catenin mutations and also for cases that are observed in the setting of hepatitis C virus infection, which show even higher rates of β-catenin gene mutations (137). In the current study, we show that Peg-IFN, a first line therapy for the treatment of hepatitis C virus infection, is capable of decreasing the transcriptional activity of β-catenin in liver tumor cells and does so by a lesser recognized but highly relevant mechanism that entails an upregulation of the β-catenin nuclear export factor RanBP3. This finding was definitively confirmed through utilization of the TOPFlash luciferase reporter assay which showed up to 50% decrease in β-catenin/Tcf mediated transcription following 4 doses of Peg-IFN (100U/ml). The significance of this observation was evident *in vivo* in DEN-exposed β-catenin TG mice, which were treated by weekly Peg-IFN and showed a dramatic decrease in cell proliferation in the liver secondary to increased total RanBP3, decreased nuclear β-catenin and cyclin D1.

Multiple negative regulators of the Wnt pathway have been identified over the last 20 years. One well-known negative regulator that is upregulated in HepG2 cells after recombinant IFN-α treatment is Dkk-1 (236). We also identified an increased expression of Dkk-1 in several hepatoma cell lines brought about by Peg-IFN induces. Dkk-1 acts to inhibit Wnt pathway

signaling by binding to LRP6 and preventing its interaction with frizzled (237, 239). Formation of this Fz-LRP6 complex is essential for canonical Wnt signaling. While an increase in Dkk-1 expression may be effective in regulating the Wnt pathway when β-catenin is not mutated, it is unlikely to affect tumor cells that harbor stabilizing mutations, such as is seen in HepG2 cells. Given this, we sought to explore additional negative regulators of the Wnt pathway, which would be more feasible as candidates to negatively regulate mutated β-catenin.

One candidate that is proven to be effective against wild-type and mutated β-catenin alike is the nuclear export factor RanBP3 (30). RanBP3 was shown to be capable of exporting from the nucleus, even the forms of β-catenin harboring mutations in exon 3. We report here that RanBP3 is a novel downstream target of Peg-IFN and a key mediator of Peg-IFN's negative impact on Wnt signaling. Qu et al had previously reported an increase in RanBP5 in response to interferon-α treatment of the HepG2 cells (236). Independently, over expression of RanBP3 in HepG2 cells, a hepatoma cell line containing stable β-catenin, mimicked the effect of Peg-IFN and inhibited Wnt-mediated transcriptional activation. RanBP3 was first identified as a chromosome region maintainence-1 (CRM1)-dependent factor involved in protein nuclear export (240). Interestingly, RanBP3 appears to act on β-catenin independently of CRM1 (30). Several other mechanisms for nuclear export of β-catenin have been previously reported including direct binding of β-catenin to the nuclear pore complex and CRM1 dependent export involving APC (reviewed in (241)). The identification of RanBP3 as a novel target of Peg-IFN could explain the partial success of the drug in affecting the incidence of HCC in the Hepatitis C patient population. Since many of the mutations in β-catenin observed in HCC occur within exon 3, a therapy effective at nuclear export of mutated β-catenin will be of clear significance in HCC treatment.

The biological effect of β-catenin inhibition by Peg-IFN was evident *in vivo* in our TG mouse model. Serine-45, a site critical for phosphorylation and eventual degradation of β-catenin (6), was mutated and over expressed in the hepatocytes. These mice lack any overt phenotype but in response to a single injection of diethylnitrosamine, exhibit hepatic tumors, which are evident in 6-month old TG mice and not in age-matched DEN-injected wild-type FVB controls. Increased hepatocyte proliferation was evident in the DEN-injected 4-month old TG livers who had received six weekly saline injections from 2.5-month of age until sacrifice. However, the TG mice receiving Peg-IFN instead of saline showed a significant decrease in hepatocyte cell proliferation, which coincided with increased RanBP3, increased association of RanBP3 and β-catenin, and decreased nuclear β-catenin, cyclin D1, and cytoplasmic GS levels.

In patients, case reports have previously shown Peg-IFN to not only be effective as part of the anti-tumor regimen for HCV-associated HCC, but also successful in chemoprevention as patients treated with Peg-IFN are less likely to develop HCC (138, 226-229, 242). We believe this effect is due at least in part to the anti-β-catenin activity of Peg-IFN via upregulation of RanBP3 as presented here. While this therapy has been used mostly for patients with HCV infection, it may have greater potential utility. Given that many patients with HCC show aberrant activation of the Wnt signaling pathway independent of HCV infection status, more patients could benefit from β-catenin targeted therapy with Peg-IFN. In addition, inclusion of Peg-IFN in loco-regional therapy for HCC such as in chemoarterial embolization may be highly efficacious and may circumvent some of the untoward side effects associated with this agent.

6.5 SUMMARY OF FINDINGS AND FUTURE DIRECTIONS

Findings

- Treatment of animals exposed to DEN with Peg-IFN decreased nuclear levels of β-catenin along with expression levels of two downstream targets, cyclin D1 and glutamine synthetase.
- Treatment of HepG2 and Hep3B cells with Peg-IFN decreased β-catenin activity as measured by the Topflash assay.
- Treatment with Peg-IFN increased expression of RanBP3 and DKK-1 in HepG2 and Hep3B cells. RanBP3 was also increased in Snu-449 and Huh-7 cells.
- Increased expression of RanBP3 led to increased association of β-catenin with RanBP3, and suppression of RanBP3 partially reversed the decrease in TopFlash activity in HepG2 cells treated with Peg-IFN.

Future Directions

- Examine the role that DKK-1 may be playing in the negative regulation of β-catenin activity by Peg-IFN.
- Examine the effect of over expression of the IFN-α gene on Topflash in human hepatoma cell lines, followed by studies using various deletion mutations in the gene in an attempt to identify a small molecule that could negatively regulate the Wnt pathway.
- Test the chemopreventative and/or chemotherapeutic benefit of Peg-IFN treatment in liver cancer models that involve β-catenin activity as a promoting factor in tumor development, such as the DEN plus Phenobarbital model or DEN induction in the S45D β-catenin TG mouse.

7.0 GENERAL DICUSSION

7.1 BETA-CATENIN IN LIVER GROWTH AND HEPATOCELLULAR CARCINOMA

The role for β-catenin in normal liver growth has been examined in previous studies. In particular, expression of β-catenin and its downstream targets coincides closely with the growth spurt that occurs in mouse liver after birth (88). Additionally, conditional deletion of β-catenin in the liver resulted in smaller liver size throughout the animal's lifespan (84). The importance of β-catenin in liver growth has also been extended to liver regeneration as an absence of β-catenin delays the regeneration process following partial hepatectomy (84, 95). From these studies, we can extrapolate that β-catenin plays an important role in hepatocyte proliferation that occurs as a significant component of these physiological growth processes in liver. It is also of significant interest that β-catenin is commonly implicated as a player in HCC. A distinct subset of HCCs displays Wnt pathway activation as a result of various mechanisms, including mutations in *CTNNB1* the gene encoding for β-catenin (123). Typically, these mutations are missense point mutations affecting key phosphorylation sites involved in β-catenin degradation with Ser45 being commonly altered (120). While multiple animal studies have been performed inducing increased activity of β-catenin in liver, no current animal study has shown spontaneous HCC in TG mice over expressing wild-type or constitutively active β-catenin (89, 90, 134). The only

model that displays spontaneous HCC is the conditional APC deletion; however APC deletions are not reported in HCC patients (131, 132). Thus, we sought to explore what effect a mutation similar to that commonly seen in patients would have on liver growth and development of HCC.

We developed a mouse transgenic model which over expresses β-catenin that is mutated at a single key phosphorylation site, Ser45, specifically in the liver. While we did see a modest increase in liver growth post-natally in these animals that seemed to be a result of increased hepatocyte proliferation, the increase in growth was not sustained beyond 2 months of age. In fact, it appears that β-catenin transgene accelerated the hepatic growth spurt and WT caught up to TG by 2 months. Furthermore, we did not observe spontaneous development of HCC as the mice were followed for up to 18 months without evidence of dysplasia. Further examination of the model to explain why over expression of mutated β-catenin did not lead to a longer term growth advantage yielded an interesting finding in that from 2 months of age excess β-catenin was sequestered at the membrane in association with both E-cadherin and Met. This suggested that the hepatocyte could compensate for the expression of "undegradeable" β-catenin by soaking up the excess at the membrane like a sponge. Further, the data argue against a role for β-catenin mutated at a single site, at least at Ser45, in the induction of liver dysplasia. Given that phosphorylation at Ser45 is the priming step for phosphorylation at the other serine/threonine sites, it is likely that mutation of those sites would yield the same results. This model will be useful to study how single site mutation leads to sequestration of β-catenin at the hepatocyte membrane.

In light of the observed membranous sequestration of β-catenin, the phenotype or lack thereof, in TG mice is not surprising. Although Ser45 mutated β-catenin did not induce spontaneous HCC, we did observe enhanced promotion of tumor growth in mice after exposure

to a chemical carcinogen, DEN. This agrees with previous studies which show that mice with deletion of β-catenin exon-3 alone lacked spontaneous tumorigenesis, but when exon-3 deletion was combined with mutation of H-ras spontaneous tumors developed (134, 135). Interestingly, it has been reported that DEN frequently induces mutations in the Ras gene so it will be important to examine Ras mutations in our model to verify cooperation between the two pathways in HCC. Thus, the current data supports that mutation in *CTNNB1* alone is insufficient to cause spontaneous HCC and suggests the requirement of a "second hit", such as chemical induction in tumorigenesis.

While it appears that the role for mutated β-catenin in HCC is via promotion of tumor growth after the initial transformation of hepatocytes, it is still unclear what players downstream of β-catenin are important in this process. The identification of these downstream effectors would provide new potential targets for therapy. In our model, we utilized a gene array approach to compare genetic changes in livers of DEN-exposed 6M-old TG and WT mice. A group of genes previously associated with HCC were upregulated, including *IGFBP5* and *JUN* oncogene (202, 203). Not surprisingly, both of these genes are regulated by β-catenin (204, 205). Also of note, *LKB1* expression was downregulated which supports a previous study showing that its loss in exon-3-deleted β-catenin over expressing mice accelerates HCC development (206). Cyclin I expression was also decreased and is a negative cell cycle regulator (207). Future studies will be important to further validate these potential targets as downstream players in tumors bearing mutations in β-catenin.

7.2 BETA-CATENIN IN OVAL CELLS AND BILIARY DISEASE

The presence of the adult hepatic progenitor cell, or oval cell, has been debated for quite some time. Early studies in rat liver found that some type of transitional cell that possessed features of both hepatocytes and cholangiocytes could generate new hepatocytes in an experimental model of HCC (243, 244). It was further suggested these transitional cells resided in the terminal bile ducts and could be activated to produce hepatocytes in some types of liver injury (168). While the terminal bile ducts are most frequently studied as the location for these cells, a more recent study identified several other potential niches for the hepatic progenitor (148). It is believed that these cells are involved in liver repair and regeneration in situations where the resident epithelial cells (hepatocytes and cholangiocytes) are incapable of completing the process due to significant cellular injury or being overwhelmed by the quantity of regeneration necessary. Many studies over the past several decades have begun to study what role these cells may have in repair of hepatic injury, as well as exploring the possibility that these cells are a player in liver tumor development. Furthermore, several groups are trying to isolate this population of cells to examine their potential for use in cell transplantation therapy as an alternative or bridge to orthotopic liver transplantation.

The “oval cell” is believed to be bipotential in that it can give rise to both hepatocytes and cholangiocytes, although the molecular mechanisms that underlie the induction, proliferation, and differentiation of oval cells are very poorly defined. Multiple studies have been done in an attempt to identify important molecular pathways. One such pathway that seems to have an important role is the Wnt/β-catenin pathway. The role for the Wnt pathway in stem cell self-renewal and adult tissue stem cells in other organs has been well studied, so it is not surprising that this pathway be involved in the activation of oval cells. Expression of active β-

catenin and its cytoplasmic/nuclear localization increase along with oval cell proliferation in the rat 2-AAF+partial hepatectomy model (177). Similarly expression of β-catenin occurs in ADP of mouse liver after DDC diet feeding (177, 178). Furthermore, the absence of β-catenin in liver leads to a blunted ADP after short-term (2-4 weeks) exposure to DDC. These findings suggested that β-catenin was playing some role in the induction and proliferation of ADP arising after DDC exposure. Despite a blunted ADP after short-term DDC feeding in the KO, we found that ductular proliferation took off dramatically in KO liver during longer DDC feeding. It is possible that some other pathway or multiple pathways could be upregulated in the absence of β-catenin much like is observed in these animals after partial hepatectomy (84). Indeed such a recurring theme of adaptive changes or compensatory pathway activation is becoming increasingly apparent in liver regeneration studies (245). Interestingly, this increase in ductular proliferation coincided with a gradual repopulation of the liver with β-catenin-positive hepatocytes which starts periportally around 30 days of DDC feeding. However, none of the ADP, oval cells, or biliary epithelial cells were ever β-catenin positive in KO throughout DDC feeding and a lack of β-catenin led to biliary dysfunction observed as intrahepatic cholestasis. How are β-catenin KO livers being repopulated by β-catenin positive hepatocytes? An initial hypothesis is that a β-catenin positive oval cell could differentiate into a hepatocyte and then repopulate the liver. This does not appear to be the source though as no A6 positive ductular cells were positive for β-catenin in the KO, thus arguing against the repopulation being an oval cell-mediated process. Another possibility to consider is transdifferentiation of one of the non-parenchymal cell types in the liver to a hepatocyte. We know that stellate cells and Kuppfer cells, as well as sinusoidal endothelial cells, retain expression of β-catenin in our KO mouse model. Another potential source for expression of β-catenin in the hepatocytes might be a fusion event between a β-catenin

negative hepatocyte and a β-catenin positive hematopoietic cell. It was previously shown that hepatocytes derived from bone marrow cells arise via fusion rather than differentiation of hematopoietic cells (246). Studies could be performed where the animal's resident bone marrow is replaced by GFP positive donor marrow cells and the study repeated to see if colocalization of GFP and β-catenin in the hepatocyte occurs. Whatever may be the source of the β-catenin positive hepatocytes, another issue that remains unresolved is that as these cells differentiate and mature into hepatocytes they should begin expressing albumin and automatically turn on cre-recombinase expression. This should theoretically lead to β-catenin deletion in these new mature hepatocytes. This suggests that β-catenin positive hepatocytes repopulating the β-catenin KO liver after long-term DDC feeding maintain incomplete maturation as was revealed by decreased expression of CEBPα, GS, and Cyp2E1. Also, these immature hepatocytes continue to proliferate and this ongoing proliferation leads to tumor formation that is fueled by ongoing injury, inflammation, and fibrosis. Finally, it has been reported that random recombination events can occur that eliminates expression of the cre recombinase. This would allow for expression of β-catenin in the hepatocytes in our model. While we cannot definitively rule this out, the fact that this process originates strictly periportally argues against this being a random event. The fact that the injury induced by DDC is primarily periportal, it is conceivable that an increase in proliferation in this region would provide an increased opportunity for recombination events specifically in this zone. Studies examining for the expression of cre in these animals will hopefully rule in or out a loss of cre as the source of β-catenin positive hepatocytes.

Expression of the ductular marker A6 is commonly observed in oval cells and cholangiocytes alike. A6 is also observed in some hepatocytes periportally after exposure to DDC diet, although no study has examined why these hepatocytes are expressing a

predominantly ductular marker. One could speculate on several possibilities for the status of these “atypical hepatocytes”. First, these cells could be hepatocytes that are newly differentiated from the bipotential oval cell and have not yet lost expression of this marker during the differentiation process. Another possibility is that periportal hepatocytes are responding to biliary injury by undergoing transdifferentiation into ductular cells and an early step in this process might be acquiring expression of A6. It has been reported that hepatocytes are capable of making the transition to cholangiocytes to replace damaged biliary epithelium. Additionally, expression of other biliary markers in hepatocytes in human biliary diseases is also reported. Thus, it is an attractive hypothesis that hepatocytes have a role in the repair of biliary injury acting as a source for new biliary epithelial cells to replace those that are lost. In our model, we found that over expression of β-catenin in hepatocytes (under albumin promoter) led to the appearance of many more atypical hepatocytes when compared to the wild-type animal and this was evident in all zones, not just periportally. This finding was coincident with a better capacity for these animals to decrease several markers of biliary injury during long-term DDC diet feeding. To test whether these animals truly had an advantage in repair of biliary injury, recovery studies were performed where mice were fed DDC for 4 weeks (a time at which the number of atypical hepatocytes is already higher in the TG mouse) and then returned to their normal chow so that they could recover from the injury. The β-catenin over expressing mice experienced more rapid serum biochemical and histological recovery in this part of the study as evidenced by improved intrahepatic cholestasis; thus, it is possible that the presence of more atypical hepatocytes had a hand in the repair process. While this finding does not definitively prove the supposition that atypical hepatocytes are acting as a source of new cholangiocytes to replace those that are injured, it does show that over expression of β-catenin is somehow providing the liver with an

increased capacity to respond to the injury that occurs after DDC diet feeding. Ultimately, lineage tracing studies will need to be performed to answer several questions relating to atypical hepatocytes. First, what is the source of these cells? Are these hepatocytes that are transitioning toward a biliary phenotype, or are they cells which have recently differentiated from the oval cell or some other yet undefined hepatic progenitor cell? Second, what is the eventual fate for atypical hepatocytes? Are they unipotential or bipotential in their differentiation capacity? Answers to these questions will provide significant advances on the understanding of progenitor cell populations in the liver. It will also be of interest to further explore why we observe more rapid decrease in injury as evidenced histologically and via serum analysis in the setting of β-catenin over expression. We also observed an increase in levels of serum bilirubin and hepatic fibrosis in the absence of β-catenin, further suggesting that β-catenin may play some role in controlling biliary injury in the DDC diet model. Several questions arise from these studies. Is the presence of more atypical hepatocytes responsible for this finding? Or is it possible that better proliferation of these atypical hepatocytes provides more cells to handle conjugation and excretion of bilirubin? From a therapeutic standpoint, it would be interesting to explore treatment with Wnt ligands or some other activator of β-catenin after biliary injury. These pre-clinical studies may provide a new therapeutic option for patients afflicted with a variety of biliary diseases. It will be important though to weigh the benefits of inducing repair of injury against the potential cost of promoting tumor development given the known role for β-catenin in hepatic tumorigenesis.

7.3 TARGETING BETA-CATENIN FOR HCC THERAPY

Due to its well-documented association and role in HCC as well as hepatoblastomas, β-catenin is an interesting, yet elusive, potential target for cancer therapy. Although drug development has been relatively slow and unsuccessful, several new compounds are in the pipeline. These include the two fungal derivatives, PKF115-854 and CGP049090, which are capable of blocking the interaction between β-catenin and TCF, and the small molecule inhibitor, ICG-001, which effectively prevents the interaction between β-catenin and the transcriptional co-activator CBP (141, 142). We report here that β-catenin is a new target for a compound that has been around for a while and used in the clinic, pegylated-interferon-α2a (trade name: Pegasys©).

The interest in Peg-IFN as a potential negative regulator of β-catenin activity came from studies showing that Hepatitis C virus (HCV) infected patients treated with Peg-IFN were less likely to develop HCC. Since many cases of HCV-associated HCC show nuclear accumulation of β-catenin, it made sense that the anti-tumor effects of Peg-IFN might be through negative regulation of β-catenin. Our studies utilizing the Topflash reporter assay in a couple human hepatoma cell lines definitively shows negative regulation of β-catenin. Another study also recently reported an effect on the pathway following IFN treatment (247). Given that Peg-IFN is already used frequently in humans and its safety profile well-defined, the finding that this drug has off-target effects on the Wnt pathway suggests that it may have utility in treatment of not only HCV patients, but also many other cases of HCC associated with β-catenin activity that are independent of HCV. Studies in animal models of HCC associated with β-catenin activation will likely provide further pre-clinical evidence in favor of using Peg-IFN or some other similar compound in a broader patient population.

While the full mechanism has yet to be elucidated, it was interesting to find that the one mechanism of action was not via an increase in degradation of the protein itself, but rather by nuclear exclusion of β-catenin by upregulation of the nuclear export factor RanBP3. This is supported by previous studies showing that RanBP3 efficiently induces nuclear export of β-catenin including a form of β-catenin with deletion of its N-terminus which is often observed in HBs and is present in the HepG2 cell line (30). While it is clear that RanBP3 induces nuclear export of β-catenin, it is not yet clear what other pathways may be affected by RanBP3. It will be important to take a more global approach to examine the impact over expression of RanBP3 would have on both normal and tumor cells. Ultimately, new therapies directed at increasing expression of RanBP3 could also prove successful in treating tumors associated with enhanced β-catenin activity.

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